Quantifying Lucerne (*Medicago sativa* L.) Genotype by Environment Interactions in the Cool Temperate Dairy Regions of Australia

by

Keith Gerard Pembleton, BAgrSc (RT) (Hons 1)

Submitted in fulfilment of the
requirements for the Degree of
Doctor of Philosophy

University of Tasmania

September 2010

DECLARATION OF ORIGINALITY

This thesis contains no material that has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and duly acknowledged in the thesis. To the best of my knowledge, no material previously published or written by another person has been used except where due acknowledgement is made in the text of the thesis. This thesis does not contain any material that infringes copyright.

Keith G. Pembleton

01/09/2010
Date

STATEMENT ON AUTHORITY OF ACCESS

Keith G. Pembleton

01/09/2010
Date

ABSTRACT

Lucerne (*Medicago sativa* L.) has a considerable amount of genetic diversity for many agronomic and physiological traits. This diversity is highlighted through the considerable genotype by environment interaction influences on yield observed in Europe, North America, and the subtropical regions of Australia. There is a need to quantify the influence of genotype by environment interactions on yield and key physiological processes in the cool temperate dairy regions of Australia. This information will ensure that appropriate cultivars can be selected and best management practices developed so that lucerne can become a greater component of the dairy feedbase.

Field experiments indentified that genotype by environment interactions occur in cool temperate regions, with winter dormant genotypes adapted to low yield potential environments, and winter active genotypes adapted to high yield potential environments. Irrigation was identified as a major management input determining genotype by environment interactions. The relative influence of each yield component was not affected by a genotype by environment interaction, and mass per shoot consistently had the greatest impact on yield accounting for up to 80% of the variability in yield.

Cultivar influenced taproot sugar and starch concentrations only with irrigation. SARDI 10 (a highly-winter active cultivar) had lower taproot sugar concentration and SARDI 7 (a winter active cultivar) had lower taproot starch concentrations than the other cultivars. When not irrigated over summer, taproot soluble protein concentrations of Grasslands Kaituna (a semi-winter dormant cultivar) were greater than SARDI 10. All cultivars had a greater abundance of vegetative storage proteins (VSPs) in taproots and enhanced phenotypic and genetic expression of winter dormancy under dryland conditions.

Glasshouse experiments revealed that increasing water deficits during regrowth decreased mass per shoot and shoots per plant. Water deficits of 75% or less of the replacement water requirement decreased total plant photosynthesis only through a reduction in leaf area and not by a decrease in either net carbon dioxide exchange rate or efficiency of photosystem II. Taproot starch concentration decreased and soluble sugar concentration increased with increasing water deficit. Plants receiving 25% of their water requirement accumulated soluble proteins seven days earlier than fully watered plants. Water deficits of 50% or less than the replacement water requirement also increased the abundance of VSPs, but VSP accumulation patterns and gene transcript levels were similar irrespective of drought treatment. With water deficit, the cold acclimation responsive gene CAR1 had a fivefold increase in expression in taproots of Grasslands Kaituna but not SARDI 10.

These experiments have shown that in the cool temperate dairy regions of Australia, under dryland conditions, the more winter dormant cultivars should be grown, while if irrigation is available, winter active cultivars should be grown. In addition this study has highlighted that, for winter dormant cultivars, cold acclimation genes impart the ability to adapt to dry conditions.

ACKNOWLEDGEMENTS

I would like to thank and gratefully acknowledge the following people and organisations:

My supervisors Dr Danny Donaghy, Dr Richard Rawnsley, Dr Jeff Volenec and Stuart Smith for their guidance, support and patience over the past four years. You have instilled in me an excitement for science and have given me a strong philosophical underpinning for a career in research.

Stuart Smith for providing me with the cultivar and experimental line data sets used in Chapter 3.

The University of Tasmania for providing me with an Australian Postgraduate Award and Dairy Australia Ltd. for providing a scholarship top up. Dairy Australia Ltd. also provided financial support for much of this research. This endeavour would not have been possible without this support.

The Australian-American Fulbright Commission for awarding me the 2008 Fulbright Tasmanian Scholarship and the Tasmanian Government and the University of Tasmania for financially supporting the Fulbright Tasmanian Scholarship. Being a Fulbright scholar was truly a great experience and one that I will cherish for the rest of my life. Also Prof. John Roche for encouraging me to apply for the Fulbright Scholarship.

The Tasmanian Institute of Agricultural Research at the University of Tasmania, Department of Agronomy at Purdue University, Tasmanian Department of Primary Industries, Water and Environment and the United States Department of Agriculture's Agricultural Research Service for providing equipment and access to field and laboratory facilities used in this research.

Peter Chamberlain, Scott Carlson, Karen Christie and the farm staff at the TIAR Dairy Research Facility and the University of Tasmania Research Farm for their

assistance in the field and glasshouse. Without your help and good humour much of this fieldwork would not have been completed.

Suzanne Cunningham for her assistance, support and patience in the laboratory components of this research. You took a field scientist with absolutely no laboratory skills and patiently imparted your knowledge and skills to me.

Dr Ross Corkrey for assistance with many of the statistical procedures used throughout the research.

Dr Allan Humphries for providing me with the information from the SARDI lucerne experimental line database that was used in this thesis, and for your advice with some of the research work. I have enjoyed our discussions and debates over the past four years and look forward to many more.

Dr Joe Anderson and Pasajee Kongsila for their assistance with the design, implementation and analysis of the qRT-PCR component of the research.

Dr Vagner Benedito and Dr Michael Udvardi for providing the list of possible reference genes for use in the qRT-PCR analysis.

Darren Driscol of Heritage Seeds and David Squib of Wrightson Seeds for very kindly providing seed that was used in the field and glasshouse experiments.

My friends and colleagues at both the University of Tasmania and Purdue University. Particularly Tom, Meisha, Mark, Chris, James and Jen. I have appreciated our discussions and debates to do with research and other matters, and your friendship and support has kept me sane through this entire process.

My friends Lynne, Kelly and Lyndsy for bringing me into your homes and giving me a family away from home during my visits to the USA.

This thesis is dedicated to my Pop, Keith Francis Pembleton. Pop you were there at the start of this journey and even though you left us part way through, I know that you are still with me here at the end.

ACADEMIC ACHIEVEMENTS

Peer-reviewed science publications from this thesis

Pembleton KG, Rawnsley RP, Donaghy DJ, Volenec JJ (2009) Water deficit alters canopy structure but not photosynthesis in regrowing alfalfa (*Medicago sativa* L.). *Crop Science* **49**, 722-731.

Pembleton KG, Volenec JJ, Rawnsley RP, Donaghy DJ (2010) Partitioning of taproot constituents and crown bud development are affected by water deficit in regrowing alfalfa (*Medicago sativa* L.). *Crop Science* **50**, 989-999.

Pembleton KG, Smith RS, Rawnsley RP, Donaghy DJ, Humphries AW (2010) Genotype by environment interactions of lucerne (*Medicago sativa* L.) in a cool temperate climate. *Crop and Pasture Science* **61**, 493-502.

Pembleton KG, Donaghy DJ, Volenec JJ, Smith RS, Rawnsley RP (2010) Yield, yield components and shoot morphology of four contrasting lucerne (*Medicago sativa*) cultivars grown in 3 cool temperate environments. *Crop and Pasture Science* **61**, 503-511.

Pembleton KG, Cunningham SM, Volenec JJ (2010) Effect of summer irrigation on seasonal changes in taproot reserves and the expression of winter dormancy/activity in four contrasting lucerne cultivars. *Crop and Pasture Science* **In Review**

Science conference oral presentations from this thesis

Pembleton KG, Rawnsley RP, Volenec JJ, Donaghy DJ, Smith S (2008) 'Yield components of contrasting alfalfa cultivars under rain-fed conditions in Tasmania, Australia', *Proceedings of the Joint Annual Meetings of The Geological Society of America (GSA), Soil Science Society of America (SSSA), American Society of Agronomy (ASA), Crop Science Society of America (CSA)*, 5-10 October 2008, Houston, Texas USA

Pembleton KG, Donaghy DJ, Rawnsley RP, Volenec JJ, and Smith S (2007) 'Yield and yield components of lucerne cultivars during establishment in three cool temperate environments', *Sharing Excellence in Research, University of Tasmania Postgraduate Research Conference*, 21 September 2007, University of Tasmania, Sandy Bay, Hobart, pp. 21.

Science conference poster presentations from this thesis

Pembleton KG, Cunningham, SM, Volenec JJ (2009) 'The effect of water deficit on the seasonal changes in taproot assimilates for 4 contrasting alfalfa cultivars in Tasmania, Australia', *Proceedings of the 2009 International Annual Meetings of the American Society of Agronomy (ASA), Crop Science Society of America and Soil Science Society of America (SSSA)*, 1-5 November 2009, Pittsburgh, PA, USA

Pembleton KG, Rawnsley RP, Donaghy DJ, Volenec JJ, Smith S (2008) 'Response of contrasting alfalfa cultivars to summer irrigation in Tasmania, Australia', *Proceedings of the Joint Annual Meetings of The Geological Society of America (GSA), Soil Science Society of America (SSSA), American Society of Agronomy (ASA), Crop Science Society of America (CSA)*, 5-10 October 2008, Houston, Texas USA

Major awards and scholarships awarded during candidature

Australian Postgraduate Award

2008 Fulbright Tasmanian Scholarship

Dairy Australia PhD scholarship top up

Minor awards awarded during candidature

2006 Australian Agronomy Conference Postgraduate Travel Award

2007 Best presenter in the Environmental Sciences, Tasmania Postgraduate Research Conference, Hobart

TABLE OF CONTENTS

COMMON ABBREVIATIONS

[^{32}P]dCTP	deoxycytidine triphosphate labelled with radioactive phophoruse-32
ANOVA	analysis of variance
bp	base pair
C	Carbon
C3	reductive pentose phosphate
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
DAE	days after emergence
DD	deep drainage
DM	dry mater
DNA	deoxyribonucleic acid
DUL	drained upper limit
EDTA	ethylenediaminetetraacetic acid
ET	evapo-transpiration
Fv/Fm	maximum efficiency of photosystem II
H_0	harvest prior to the initiation of water treatments
K	Potassium
LSD	least significant difference
MgCl	magnesium chloride
Mo	Molybdenum
MSC	mean stage count
MSW	mean stage weight
MtGEA	*Medicago truncatula* Gene Expression Atlas
N	Nitrogen
NaCl	sodium chloride
NaH_2PO_4	monobasic sodium phosphate
Na_2HPO_4	dibasic sodium phosphate

P	Phosphorus
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
SARDI	South Australian Research and Development Institute
SDS	sodium dodecylsuphate
SDS-PAGE	sodium dodecylsuphate polyacrylamide gel electrophoresis
SSC	sodium chloride - sodium citrate buffer
SWC	soil water content
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween 20
TNC	total nonstructural carbohydrate
VSP	vegetative storage protein

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF EQUATIONS

CHAPTER 1

Introduction

The profitability of dairy farm businesses is strongly correlated to the amount of "home grown" forage consumed by the milking herd (Doonan 2003; Dillon *et al.* 2005). As such, the management of the forage base on dairy farms is a key determinant of profitability (Dillon *et al.* 2005). Australian dairy farms utilise a range of forage species including a mixture of perennial pastures and crops (Table 1.1). In the temperate dairy regions of Australia (south eastern mainland Australia and Tasmania), the forage base is predominantly perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) pasture, with forage crops such as brassica species (*Brassica* spp.) utilised as part of a pasture renovation program (Pritchard *et al.* 1991; Read *et al.* 1991; Fulkerson and Doyle 2001). However, the warm dry summers experienced throughout the temperate dairy regions of Australia limit the production of dryland pastures (Rawnsley *et al.* 2007) and, in many areas, government policy limitations to the availability of irrigation water restrict further expansion of irrigated land.

Across the Australian agricultural sectors, there is increasing interest in the use of lucerne (syn alfalfa; *Medicago sativa* L.) as a means of 'drought proofing' pastoral enterprises, as well as to provide soil amelioration and salinity control in cropping systems (Robertson 2006). Lucerne has superior nutritive value compared to temperate grasses at a similar growth stage (Frame and Laidlaw 2005), a complementary growth pattern to dryland and irrigated temperate grasses (Douglas 1986; Brown *et al.* 2005a), a longer growing season than temperate grasses and annual legumes under dry summer conditions (Douglas 1986; Brown *et al.* 2005a), and greater flexibility in utilisation (i.e. direct grazing, or conserving as hay or silage; Frame *et al.* 1998). Further to this, the major historical limitations to an increased use of lucerne in Australian pastoral systems (e.g. pest and disease pressures, soil acidity and intermittent water logging) have been overcome by an improved understanding of the plant's response to biotic and abiotic stresses. This has led to

the development of improved cultivars and better crop management practices (Auricht 1999; Lowe *et al.* 2002; Grewal and Williams 2003; Humphries *et al.* 2006a; Robertson 2006).

Table 1.1. Current forage base of the Australian Dairy Industry by climatic region. Adapted from Kelly *et al.* (1991), Pritchard *et al.* (1991), Read *et al.* (1991) and Fulkerson and Doyle (2001).

Region	Geographic locations	Major pastures utilised	Forage crops
Cool temperate	Tasmania, southern Victoria, south east coast of New South Wales.	Perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.).	Forage brassicas (*Brassica* spp.).
Mediterranean	Southwest Western Australia, southeast South Australia, northeast Victoria, southwest Victoria.	Dryland: Annual ryegrass (*Lolium. multiflorum* Lam.) and subterranean clover (*Trifolium. subterraneum* L.). Irrigated: Perennial ryegrass, white clover, paspalum (*Paspalum dilatatum* Poir.) and kikuyu (*Pennisetum clandestinum* Hochst. ex Chiov.).	
Inland Irrigation	Goulburn and Murray valleys and Maffra area of Victoria, Riverina of New South Wales.	Perennial ryegrass, white clover and paspalum.	Forage maize (*Zea mays* L.) and Sorghum (*Sorghum bicolor* L.), millets (*Echinochloa utilis* Ohwi. *Pennisetum glaucum* L.) and forage brassicas.
Subtropical	Queensland and New South Wales north coast.	Perennial ryegrass and annual ryegrass, paspalum, rhodes grass (*Chloris gayana* Kunth), kikuyu, white clover and lucerne (*Medicago sativa* L.).	Forage maize, sorghum and oats (*Avena sativa* L.).

Considerable genetic diversity exists within the lucerne species (Quiros and Bauchan 1988) which has resulted in the development of a diverse range of cultivars adapted to particular farming systems and environments. Evaluation of lucerne genotypes in Australia has focused on Mediterranean and subtropical broad acre

cropping and extensive pastoral systems (Lloyd *et al.* 1985; Lowe *et al.* 1985; Humphries and Hughes 2006; Humphries *et al.* 2006b). Vast differences in farming systems and the possibility of genotype by environment interactions limits the application of this work to dairy systems in the cool temperate regions of Australia. This thesis presents a body of work that aims to evaluate the genotype by environment and genotype by management interactions on lucerne grown in a cool temperate climate. Through these evaluations the suitability of lucerne genotypes for use as part of the dairy forage base in the cool temperate regions of Australia will be determined.

Specifically Chapter 2 will undertake an extensive review of literature describing lucerne growth and development and how genotype influences these processes in a range of environments. Through this review a series of hypothesis surrounding the expression of genotype by environmental interactions in cool temperate climates will be developed. Chapters 3 and 4 will describe experiments directly investigating the effect of genotype by environmental interactions on lucerne growth and development in the cool temperate regions of Australia. Chapter 5 will describe plant tissue sampling and analysis that investigates how genotype and irrigation interact to affect key taproot physiological processes. Chapters 6, 7 and 8 will describe a glasshouse experiment investigating the growth, development and key physiological processes during a regrowth cycle of two contrasting lucerne cultivars exposed to a range of water deficits. In Chapter 9, the findings of Chapters 3 to 8 will be brought together to address the hypothesis presented in Chapter 2.

CHAPTER 2

Review of Literature

2.1. Scope of review

The use of lucerne as a dairy forage in the cool temperate regions of Australia is currently limited and there is potential for it to play a substantially greater role in the future. This review will outline the current global distribution and genetic diversity of lucerne and its current role in Australian pastoral and cropping systems. It will demonstrate that the historical barriers to the inclusion of lucerne within the dairy forage base have been overcome through a better understanding of stress physiology and the release of improved cultivars. A detailed discussion of the response of plant growth and development to environmental stimuli and management practices, as well as how these responses differ amongst lucerne genotypes, will demonstrate that an evaluation of genotype by environment interactions in the cool temperate dairy regions is required. If lucerne is to become a significant component of the dairy forage base in the cool temperate regions of Australia, this information will be crucial to cultivar selection and to the development of best management practices.

2.2. Lucerne - global production and role in pastoral and cropping systems

2.2.1. History and global distribution

Lucerne is the world's oldest named forage crop, with the first recorded reference in writings from the Hittites in the Middle East being dated to between 1400 and 1200 B.C. (Russelle 2001). Between 500 B.C. and 400 A.D. lucerne spread throughout Europe and northern Africa and into Asia along trade routes and with the movement of armies (Michaud *et al.* 1988). In addition, evidence exists of Asian civilisations actively seeking out the crop when acquiring cavalry and chariot horses from the Middle Eastern empires, further increasing its distribution (Hendry 1923). The Roman Empire is credited with identifying some key lucerne management principles (Ahlgren 1949), with Pliny the Elder's writings including directions on appropriate

defoliation management, soil liming and irrigation practices to maximise production (Bolton 1962). After the fall of the Roman Empire, references to lucerne disappear from the historical record (Hendry 1923), until it was "rediscovered" in Spain in the 16th century. From Spain it spread back throughout Europe and into Russia (Michaud *et al.* 1988). At the same time, lucerne was first introduced into the Americas with Portuguese and Spanish colonisation. Lucerne was introduced into Australia and New Zealand in the early 1800s, although there is debate as to whether this introduction was sourced from Europe or Argentina (Palmer 1967). Globally, the total area of lucerne production is 32.3 million hectares (Russelle 2001). In Australia 567,000 hectares are planted to pure lucerne swards (Commonwealth of Australia 2002) and the total area planted to lucerne either as pure or mixed swards is approximately 3.5 million hectares (Kemp and Michalk 2005). The current area of production represents only a small proportion of the land suitable for lucerne with Hill (1996) estimating that 86.4 million ha in south eastern Australia (south eastern Queensland to Tasmania) is capable of supporting lucerne production. More recently Roberson (2006) suggested that 22.6 million ha of cropping land in mainland south eastern Australia is capable of supporting lucerne production. Clearly there is scope to expand the use of lucerne within Australian agricultural enterprises.

2.2.2. Species complex, genotypes and genotype by environmental interactions

The lucerne species is made up of a complex of eight subspecies which are *sativa*, *falcata*, *glutinosa*, *coerulea*, *varia*, *hemicycla*, *polychroa* and *tunetana* (Quiros and Bauchan 1988). There are two ploidy levels within the complex, with both diploids (2*x*) and tetraploids (4*x*) naturally occurring (Quiros and Bauchan 1988). This difference in ploidy level presents the main barrier to genetic exchange between the subspecies. However, this is overcome by diploids producing unreduced gametes (Irwin *et al.* 2001). Globally, of the eight subspecies, *sativa* and *falcata* have been the focus of cultivar development and commercial production (Irwin *et al.* 2001). Even though commercial cultivar development has been limited mainly to two subspecies, considerable amounts of genetic diversity for yield, plant morphology

and nutritive value traits are available in released cultivars alone, without having to source the more distantly related subspecies (Julier *et al.* 2000).

Lucerne cultivars can be grouped based on the amount of autumn and winter growth. This phenomenon is termed winter activity (or fall dormancy in North America). Lucerne cultivars are assigned a rating between 1 and 11 based on the rate of shoot elongation in late autumn (Teuber *et al.* 1998). This winter activity rating is related to the original germplasm source, and is strongly correlated to plant survival when exposed to the freezing temperatures associated with North American winters (Sheaffer *et al.* 1992). Lower scoring, winter-dormant germplasm typically originate from northern Europe and Russia and predominantly contain subspecies *falcata* in their genetic pedigree. Intermediate, semi winter-dormant scoring germplasm originates from southern Europe and the Mediterranean and contains a mixture of both subspecies *falcata* and *sativa*. Higher scoring, highly winter-active germplasm originates from Africa and India and predominantly contains subspecies *sativa* in their pedigrees (Barnes *et al.* 1977; Barnes *et al.* 1988).

The genetic diversity within the lucerne species complex has resulted in the development of a diverse range of cultivars adapted to particular environments and farming systems. This specific adaption is expressed as genotype by environmental interactions and is the result of interactions between a plant's genes and the environment in which it is grown (Annicchiarico and Piano 2005; Cooper *et al.* 2006). Genotype by environmental interaction is greatest for quantitative characteristics such as yield and quality (Wright *et al.* 1996) and when there is there is little genetic diversity within a genotype (Annicchiarico 2009). Environment has a strong influence over the expression of genotype by environmental interactions for complex traits, and as such, it is important to identify what specific environmental factor is responsible for phenotypic expression and the effect of genotype in mediating this response (Wade *et al.* 1996; Cooper *et al.* 2006; Annicchiarico 2009). Simple traits like disease and pest resistance which are controlled by few genes (Halloran and Luckett 1993) have a greater genotype control over their expression and consequently are stably expressed over a broad range of environments.

Genotype by environmental interactions for lucerne has been demonstrated using artificial environments by Annicchiarico and Piano (2005), and has been observed under field conditions by Daday *et al.* (1961), Rogers (1961) and Lamb *et al.* (2006). Genotype by environmental interactions are considered an annoyance by plant breeders as it interferes with the assessment of the genetic variation for a given trait within a population (Ceccarelli et al. 1994; Annicchiarico 2009). However, even with environment maintaining the majority of influence over the more complex traits there is still potential for the exploitation of genotype by environmental interactions by plant breeders by the development of locally adapted cultivars (Ceccarelli et al. 1994; Ceccarelli 1996; Annicchiarico and Piano 2005; Cooper *et al.* 2006; Annicchiarico 2009). and by producers through cultivar selection and genotypic specific management practices (Daday *et al.* 1961; Rogers 1961).

There are two types of genotype by environmental interactions, repeatable and non-repeatable interactions (Cooper *et al.* 2006; Annicchiarico 2009). The probability of genotype by environmental interactions for a given trait not being repeatable increases as the complexity of the trait increases (Cooper *et al.* 2006). To be able to exploit and gain full benefit, any potential genotype by environmental interactions they must be repeatable in a manner that can be forward predicted by the producer at planting time (Annicchiarico 2009).

2.2.3. Current role and uses in Australian farming systems

Lucerne is used in both intensive and extensive agricultural systems across Australia. It is used as conserved forage (primarily hay) or is directly grazed, and has a long-standing role as a ley pasture species in rotational dryland cropping systems (Lodge 1991). In both cropping and pastoral farming systems, the benefits of growing lucerne centre on its growth pattern and superior forage quality.

The growth pattern of lucerne makes it appropriate to use as a forage crop to extend the growing season and fill summer feed gaps that are associated with grass-based dryland pasture systems (Brown *et al.* 2005a). As such, lucerne is grown to

complement grass-pasture based systems in New Zealand environments similar to the southern regions of Australia (Douglas 1986; Brown *et al.* 2005a).

Compared to temperate grasses, lucerne is better quality forage. Cell wall contents and fibre fractions are less, while crude protein levels are greater than grasses at a comparable growth stage (Campling 1984). With the exception of sodium, mineral concentrations are greater than grasses at a similar stage of growth (Frame *et al.* 1998), while the amino acid profile is similar to that of temperate perennial grasses (Balde *et al.* 1993). This higher quality forage translates into increased production from animals fed lucerne instead of grass or grass/clover mixtures (Bryant *et al.* 1961; Bryant 1978; Conrad *et al.* 1982).

In addition to its superior nutritive value and complementary growth pattern, other benefits of growing lucerne include improved soil structure through increased porosity, increased aggregate stability and soil water holding capacity (Pratley and Corbin 1993), increased soil nitrogen (N) status through a symbiotic relationship with soil-borne *Rhizobia* bacteria capable of fixing between 31 to 128 kg/ha/year of atmospheric N (Peoples and Baldock 2001), a slowing of soil acidification rates by recovering leached nitrate (Bellotti *et al.* 1998) and a lowering of soil water tables by up to three metres (Ridley *et al.* 1998; Humphries and Auricht 2001).

Recently there has been interest in utilising lucerne to control ground water tables in an effort to slow the encroachment of soil salinity (Humphries and Auricht 2001; Kemp and Michalk 2005; Ward and Micin 2006). The deep-rooted nature of lucerne means that it is capable of extracting water below a soil depth of 2 m (Humphries and Auricht 2001; Lodge *et al.* 2010), and this, along with its perenniality, means that it is able to utilise summer rainfall that annual winter cereals and annual temperate pastures cannot, thereby reducing recharge into aquifers (Humphries and Auricht 2001; Ward and Micin 2006; Dear *et al.* 2010).

Despite its acknowledged benefits and high forage quality, lucerne has not been widely used within Australian dairy systems. The regions where it is a major component of the forage base are limited to the subtropical dairy regions of Queensland and New South Wales (Read *et al.* 1991; Fulkerson and Doyle 2001).

Producers consider that, as a forage species, lucerne is more difficult to manage than the more widely used perennial ryegrass (*Lolium perenne* L.; Kemp and Michalk 2005). Where lucerne is utilised, winter-active cultivars are favoured (Read *et al.* 1991) as their growth pattern better complements other forages in the forage base as well as the feed requirements of a dairy herd than the growth of winter-dormant cultivars (Irwin *et al.* 2001). However, no assessment of the suitability of specific genotypes has been made in the temperate dairy regions of southern Australia.

2.2.4. Limitations to use within the dairy forage base

2.2.4.1. Anti-quality

The risk of bloat is probably the most well known of the anti-quality factors associated with lucerne and other temperate forage legumes. While the economic impact of bloat on Australian agricultural production is unknown, its impact is considered to be significant, with the loss of thousands of cattle annually (Blair 1997; Moate *et al.* 1997). Bloat is caused by the retention of fermentation gases through the formation of a stable foam in the reticulum (Clarke and Reid 1974; McDonald *et al.* 2002). This causes the rumen to swell, with gas placing pressure on the heart and lungs, which ultimately leads to death by cardio-respiratory failure (Clarke and Reid 1974). The high concentration of soluble proteins present in the leaves of lucerne is thought to play a role in the formation of the foam that traps the gas (McDonald *et al.* 2002). Bloat can also have a subclinical effect which may go undetected. Dougherty *et al.* (1992) showed that subclinical bloat limited grazing time and negatively affected animal production.

Cattle are most susceptible to bloat when introduced to young lucerne or to lucerne for the first time. The risk of bloat can be reduced via a number of methods, either by including a high fibre component (e.g. cereal or grass hay) in the diet, by feeding the crop when it is at a more mature growth stage, utilising lucerne as a component in a species mixture, or by culling animals that show a chronic susceptibility to bloat (Frame *et al.* 1998). The use of anti-foaming agents, through drenching or rumen capsules, has been shown to be effective in protecting grazing cattle from bloat (Moate *et al.* 1997).

High phytoestrogen content in forage legumes in the *Medicago* genus can be a serious issue when feeding these species to female sheep during preparation for breeding (McDonald 1995). Phytoestrogen content in lucerne varies with cultivar and stage of regrowth (being the greatest at the early bud stage). Their concentration in the herbage also increases in response to attack by aphids and fungal pathogens (Frame *et al.* 1998). Despite their negative impact on the fertility of smaller classes livestock, phytoestrogen are not an issue in cattle (McDonald 1995).

2.2.4.2. Soil acidity and aluminium toxicity

Lucerne is not tolerant of soil acidity and the mineral toxicities and deficiencies associated with low soil pH (Grewal and Williams 2003). This intolerance represents a significant barrier to the production and utilisation of lucerne. In North America and Europe, soil pH values (in water) less than 6.5 are considered outside the optimum range for lucerne production (Frame *et al.* 1998; McGraw and Nelson 2003), while in Australia, yield is reduced when soil pH values (in water) are below 5.5 to 5.7, and establishment is seriously compromised below 4.8 (Knox *et al.* 2006; Robertson 2006). Applying lime to raise soil pH from a suboptimal to an optimal level increases forage of lucerne yields by between 11 to 32% (Grewal and Williams 2003).

Soil acidity affects both root and shoot growth. It reduces both the activity and the population of *Rhizobia* (Howieson and Ewing 1986), and increases the concentration of hydrogen ions and the associated soil nutrient deficiencies and toxicities (Foy 1984; Mugwira and Haque 1993; Baligar *et al.* 1997). The main nutrient toxicities associated with soil acidity are aluminium and manganese. Both these toxicities cause root swelling and abnormal branching while inhibiting secondary growth of the taproot (Joost and Hoveland 1986). Some reports suggest that the main limitation of acidity is from aluminium and manganese toxicity, and that, without toxic levels of these minerals, lucerne growth is relatively unaffected by acidic soils (Macleod and Jackson 1965; Munns 1965; Helyar and Anderson 1970).

Soil acidity at depth, even after surface soil acidity is corrected, presents a significant barrier to lucerne production (Douglas 1986; Carter and Richards 2000; Grewal and Williams 2003) as it prevents taproot growth into the soil profile (Carter and Richards 2000). This limitation to taproot growth prevents extraction of soil water at greater depths than other field crops and forage species, thus nullifying a major advantage of growing lucerne over other species.

Genetic variation exists in tolerance to acidity and the associated nutrient toxicities (Grewal and Williams 2003), and this diversity is being exploited by plant breeders and molecular geneticists to develop acid-tolerant lucerne cultivars (Humphries *et al.* 2006a; Khu *et al.* 2009). In addition to breeding for tolerance to soil acidity, the application of lime is common practice when growing lucerne on acid soils as it improves all facets of lucerne growth (Grewal and Williams 2003). While it is an expensive practice, if soil acidity at depth is a limitation, deep soil cultivation at the time of lime application, will improve lucerne production (Carter and Richards 2000).

2.2.4.3. Waterlogging

Many soils in the dairy regions of southern Australia are periodically waterlogged (Lewis *et al.* 1991). Lucerne is sensitive to waterlogging (Humphries and Auricht 2001). This limits its utilisation by producers, and is a serious impediment to its production worldwide (Smethurst *et al.* 2005). Waterlogging impacts on lucerne growth through a multitude of mechanisms. It reduces root growth and nutrient uptake (Barrett-Lennard *et al.* 1988; Gibbs and Greenway 2003), interrupts cold acclimatisation and winter hardening (McKenzie *et al.* 1988), prevents aerobic respiration in the roots and causes anaerobic respiration that produces ethanol and other toxic substances (Barta 1988b; Gibbs and Greenway 2003), prevents the synthesis of amino acids and proteins (Barta 1988b) and causes stomatal closure leading to reduced photosynthesis (Cameron 1973; Alva *et al.* 1985). These changes in plant physiology lead to reduced growth and root death (Smethurst *et al.* 2005), ultimately causing plant death (Humphries and Auricht 2001).

Lucerne can adapt to waterlogged conditions via anatomical modifications. This includes changes in the distribution of lateral roots and the development of internal aeration structures (aerenchyma; Zook *et al.* 1986). A gradual increase in the water content of the soil that the roots contact is required to stimulate the development of root morphological adaptations in plants (Luxmoore and Stolzy 1969; Pradhan *et al.* 1973). Humphries and Auricht (2001) suggested that the rapid submersion of the root system, which occurs in many pot experiments screening for water logging tolerance in lucerne, has not allowed the development of these root adaptations. As such, this method of screening for selection of water logging tolerance may have discarded germplasm that would have shown tolerance to water logging under field conditions.

The status of plant carbohydrate reserves has been shown to influence the tolerance of lucerne to waterlogging. Plants with higher concentrations of soluble carbohydrate in the taproot are at a greater risk of damage than plants with low soluble sugars concentrations (Lowe *et al.* 2002). Correct timing of defoliation around periods of water logging can affect the extent of plant damage. Defoliation immediately before and at the onset of waterlogging increases the amount of soluble sugars in the taproot, which leads to increased root damage, while defoliation several weeks prior to water logging reduces the concentration of soluble sugars at the onset of waterlogging and hence reduces plant damage (Barta 1988a; 1988b).

2.2.4.4. Pests

Pests of lucerne include mites, insects and nematodes. There are 16 known pests that have an economic impact on lucerne forage production in the southern regions of Australia (Table 2.1). Of these, nematodes (*Ditylenchus dipsaci* and *Meloidogyne incognita*), aphids (*Therioaphis trifolii*, *Acyrthosiphon kondoi* and *Acyrthosiphon. pisum*) and to some extent red-legged earth mite (*Halotydeus destructor*) and lucerne flea (*Sminthurus viridis*) are the pests that have the greatest economic impact (Humphries and Auricht 2001). Pests have a detrimental effect on the productivity of lucerne by slowing plant growth and development, hindering seedling establishment, acting as vectors for viral infection, damaging plant tissue, creating

entry points for bacterial and fungal infection and in extreme infestations, killing the plant (Frame *et al.* 1998).

While applications of chemical agents may be an economical method to alleviate pest pressure in cases of severe infestation, non-chemical methods are usually favoured due to lower associated costs. Scheduling harvests so that the pest's life cycle is interrupted reduces the pest on the crop during subsequent regrowth periods (Frame *et al.* 1998). Genetic resistance to many pests is available in released cultivars, or genetic material with resistance is available in germplasm banks around the world (Humphries and Auricht 2001). Breeding for aphid resistance has been an objective of the Australian lucerne breeding program since aphids were first identified in Australia (Auricht 1999). This has resulted in the release of many aphid-resistant cultivars adapted to Australian conditions (Auricht 1999; Lloyd *et al.* 2002).

Table 2.1. Pests of lucerne recognised as having an economic impact on production in the southern regions of Australia.

Common Name	Scientific Name	Reference
Spotted alfalfa aphid	*Therioaphis trifolii*	(Gramshaw 1981; Lehane 1982; Ryley 2002)
Blue alfalfa aphid	*Acyrthosiphon kondoi*	(Gramshaw 1981; Lehane 1982; Ryley 2002)
Pea aphid	*Acyrthosiphon pisum*	(Lehane 1982; Ryley 2002)
Cutworms	*Agrotis* spp.	(Kaehne and Lake 1982; Ryley 2002)
Red-legged earth mite	*Halotydeus destructor*	(Kaehne and Lake 1982)
Lucerne flea	*Sminthurus viridis*	(Kaehne and Lake 1982)
Sitona weevil	*Sitona discoideus*	(Kaehne and Lake 1982)
Wingless grasshopper	*Phaulacridium vittatum*	(Kaehne and Lake 1982)
Two-spotted mite	*Tetranychus urticae*	(Kaehne and Lake 1982)
Alfalfa leaf roller	*Merophyas divulsana*	(Gramshaw 1981; Kaehne and Lake 1982; Ryley 2002)
Lucerne leaf hopper	*Austroasca alfalfae*	(Ryley 2002)
Vegetable leaf hopper	*Austroasca viridigrisea*	(Ryley 2002)
Lucerne crown borers	*Zygrita diva* and *Corrhenes stigmatica*	(Ryley 2002)
White-fringed weevil	*Graphognathus leucoloma*	(Ryley 2002)
Stem Nematode	*Ditylenchus dipsaci*	(Kaehne and Lake 1982)
Root knot Nematode	*Meloidogyne incognita*	(Kaehne and Lake 1982)

2.2.4.5. Diseases

Fungal, bacterial and viral agents have been shown to affect lucerne production. They decrease production by reducing herbage yield and plant persistence (Summers and Gilchrist 1991), lowering herbage quality, increasing herbage anti-quality factors (Lenssen *et al.* 1991), and preventing acclimation to adverse environmental conditions (Richard and Martin 1993).

There is conflicting information on the role of plant pathogens in the decline of lucerne persistence. In lucerne crops up to two years of age, Summers and Gilchrist (1991) attributed the majority of plant death to interplant competition, with minimal influence from disease. However, in locations where pathogens that cause either phytophthora or aphanomyces root rots (*Phytophthora medicaginis* and *Aphanomyces euteiches* respectively) were present, Wiersma *et al.* (1995) found that cultivars resistant to either of those diseases had a greater chance of successful establishment. For established lucerne crops, Jones *et al.* (1984) and Smith and Bouton (1993) found that persistence was not directly associated with pathogen resistance. However, other reports have estimated that plant losses in established crops due to biotic factors are as high as 22% (Lowe *et al.* 1985; Lowe *et al.* 1987; Summers and Gilchrist 1991). There are twenty diseases of lucerne currently recognised in Australia (Table 2.2).

Fungal diseases can be controlled by chemical applications. However, this option is generally not cost-effective (Frame *et al.* 1998). Disease incidence can be reduced by maintaining good plant nutrition, reducing exposure to abiotic stress, controlling pests (as these are vectors for infection), using crop rotations to break disease cycles, ensuring good soil drainage and using genetically resistant cultivars (Frame *et al.* 1998). Tillage can also reduce the populations of some soil-borne fungal pathogens like *Rhizoctonia solani*, which causes Rhizoctonia root canker (Pumphrey *et al.* 1987). However, the perennial nature of lucerne limits this practice to before planting. Genetic resistance to many diseases is available in released cultivars or in material stored in germplasm banks around the world (Humphries and Auricht 2001). Resistance to *P. medicaginis* and *Cercospoa trifolii* (which causes southern anthracnose) has been the focus of Australian disease resistance screening

and breeding efforts (Rogers 1981; Auricht 1999), and most Australian cultivars have at least moderate resistance to both those diseases (Auricht 1999; Lloyd *et al.* 2002).

Table 2.2. Diseases of lucerne present in the southern regions of Australia.

Disease	Agent(s)	Type	Reference
Bacterial wilt	*Clavibacter michiganense* ssp. *insidiosus*	Bacteria	(Lehane 1982)
Southern anthracnose or Colletotrichum crown rot	*Colletotrichum trifolii*	Fungi	(Gramshaw 1981; Kaehne and Lake 1982)
Rhizoctonia root canker	*Rhizoctonia solani*	Fungi	(Gramshaw 1981; Kaehne and Lake 1982; Ryley 2002)
Phytophthora root rot	*Phytophthora medicaginis*	Fungi	(Gramshaw 1981; Kaehne and Lake 1982; Ryley 2002)
Common root rot	*Stagonospora meliloti* or *Acrocalymma medicaginis*	Fungi	(Kaehne and Lake 1982; Ryley 2002)
Common leaf spot	*Pseudopeziza medicaginis*	Fungi	(Kaehne and Lake 1982; Ryley 2002)
Pepper spot or Leptosphaerulina leaf spot	*Leptosphaerulina trifolii*	Fungi	(Kaehne and Lake 1982; Ryley 2002)
Phoma (black stem)	*Phoma medicaginis*	Fungi	(Kaehne and Lake 1982)
Crown, root and stem rot	*Phoma* spp.	Fungi	(Kaehne and Lake 1982)
Seedling blight	*Pythium* spp., *Rhizoctonia solani*, *Phytophthora medicaginis* and *Aphanomyces euteiches*	Fungi	(Ryley 2002)
Alfalfa mosaic virus		Virus	(Ryley 2002)
Bacterial leaf and stem spot	*Xanthomonas campestis pv. alfalfae*	Bacteria	(Ryley 2002)
Witches broom		Phytoplasma	(Ryley 2002)
Stemphylium leaf spot	*Stemphylium vesicarium*	Fungi	(Ryley 2002)
Rust	*Uromyces striatus*	Fungi	(Ryley 2002)
Downy mildew	*Peronospora trifoliorum*	Fungi	(Ryley 2002)
Cercospora leaf spot	*Cercospora medicaginis*	Fungi	(Ryley 2002)
Violet root rot	*Helicobasidium purpureum* anamorph	Fungi	(Ryley 2002)
Fusarium wilt	*Fusarium oxysporum* f.sp. *medicaginis*	Fungi	(Ryley 2002)
Sclerotium blight and Sclerotinia rot	*Sclerotium rolfsii* and *Sclerotinia trifoliorum*	Fungi	(Ryley 2002)

2.2.4.6. Persistence in grazing systems

Grazing exposes the plant to the stress of defoliation (complete removal of shoots) and also to potential damage due to tugging, trampling and animal waste excretion (Smith *et al.* 1989). The capacity of the plant to survive defoliation is determined by its ability to regrow.

Regrowth in lucerne is supported by root carbohydrate and N reserves (Avice *et al.* 1996a), and the source of the regrowth is from buds on the crown and auxiliary nodes (Leach 1968; 1969). As such, defoliation should coincide with a time when the plant has buds ready to elongate and has adequate reserves to support regrowth.

Several methods for scheduling defoliations have been devised. Over 2000 years ago in the Roman Empire, harvesting at early flowering was used (Bolton 1962), and this is still recommended practice for many environments (Sheaffer *et al.* 1988). This method gives a compromise between yield, persistence and nutritive value (Lodge 1991). In Australia, best management practice for defoliation of lucerne is to time defoliations to coincide with when crown buds are elongating (Gramshaw *et al.* 1981; Gramshaw *et al.* 1993; Lowe *et al.* 2002). Defoliating at crown bud elongation ensures not only that taproot reserves are adequate to support regrowth but in addition that there is a large number of sites available for the rapid formation of new shoots (Leach 1968; Gramshaw *et al.* 1993).

Grazing duration determines the level of exposure of the plants to tugging, trampling and waste excretion by grazing animals. Multiple attempts to identify the maximum duration of grazing before plant persistence is compromised have identified considerable environment and management effects on the length of grazing possible (Leach 1968; McKinney 1974; Janson 1975; Leach 1979). These studies have all identified that rotational grazing with short grazing durations are more conducive to persistence than longer durations. Further to this, for the first three years of production, rotational grazing (with grazing durations determined by forage on offer), maintains plant populations at levels similar to mechanically harvested crops (Lodge 1985).

Meeting the plants requirement of appropriately timed defoliations with a short grazing duration requires intensive subdivision of grazing areas to create a large number of small paddocks (Lodge 1991). This is reflective of modern Australian dairy grazing systems (Grainger *et al.* 1991; Fulkerson and Doyle 2001) and should be easy to implement on Australian dairy farms. However, in extensive grazing or ley farming systems, large paddock sizes and a limited number of

paddocks limits the application of recommended best management (Lodge 1991). Extensive breeding and evaluation efforts have been undertaken in Australia (Leach 1979; Kaehne *et al.* 1993; Humphries *et al.* 2006b) and overseas (Smith *et al.* 1989; Smith and Bouton 1993; Bouton and Smith 1998; Bouton and Gates 2003; Pecetti *et al.* 2008), with the aim of improving the persistence of lucerne subjected to extended or continuous grazing. Recently, Humphries *et al*. (2006b) suggested that a two paddock system of grazing lucerne with 5 month grazing durations may be a viable alternative to rotation grazing, as the majority and most rapid loss of plants occurs after the initial five months.

Recent advances in crop agronomic practices and plant breeding have lowered the historical barriers to the adoption of lucerne in Australian agricultural systems. The large degree of genetic diversity present between lucerne cultivars and the associated genotype by environment interactions means that careful evaluation of the preference shown for winter-active types of lucerne in the northern dairy regions of Australia will be required if this recommendation is to be translated to the southern temperate dairy regions. There is the potential to exploit genotype by environmental interactions to maximise lucerne production within southern Australian dairy systems. Traits such as yield, persistence and nutritive value are quantitative and complex by nature. As such, a thorough understanding of the genotypic and environmental influences on plant morphology, development and key physiological processors will be required to determine if the expression of these traits are repeatable across multiple locations in cool temperate Australia. By developing a thorough understanding of the physiological basis of the genotype by environmental interaction influencing these traits recommendations can be made as to which genotypes are the most appropriate for the chosen environment.

2.3. Lucerne morphology and key physiological processes

The lucerne plant is comprised of three distinct structures; shoots, a crown and a root system (Plate 2.1). The following sections will describe the morphology, development and key physiological processes of each of these plant parts and the influence of genotype upon them.

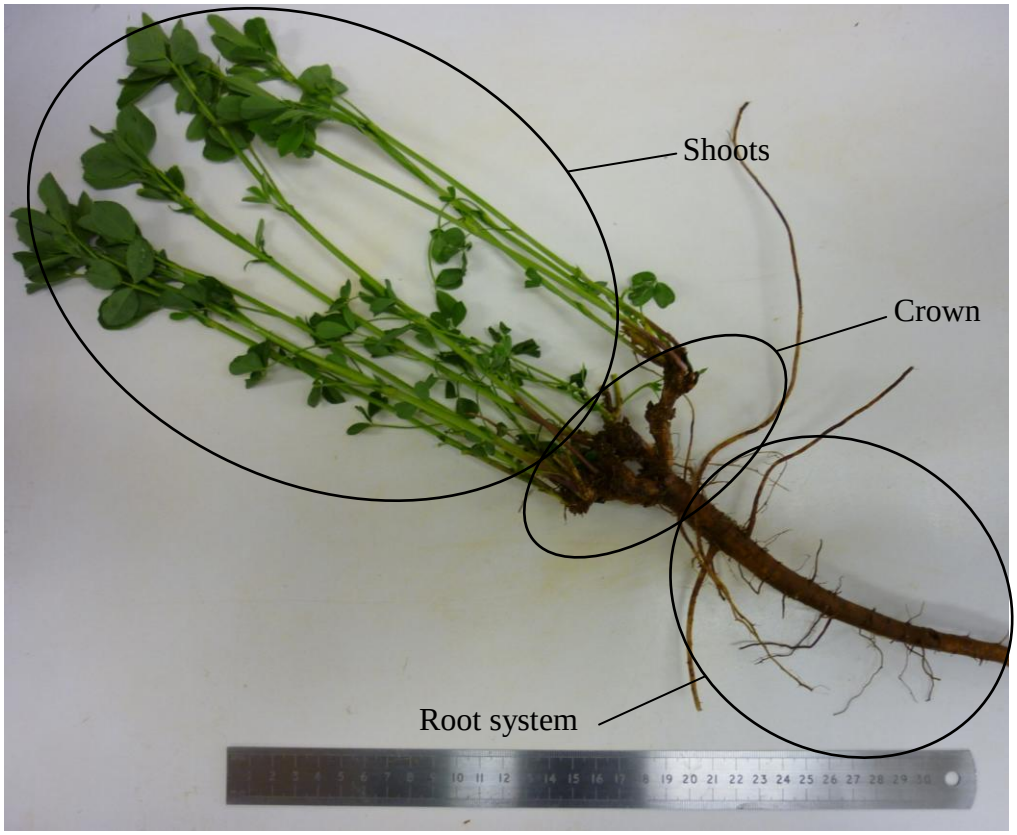

Plate 2.1. A three year old lucerne plant with the distinction between shoots, crown and root system highlighted.

2.3.1. Shoots and leaves

2.3.1.1. Morphology

Shoots are the above ground part of the plant and are the component harvested for forage. Shoots are upright and comprise leaves attached to a stem. Leaves are formed at the shoot apex by the shoot apical meristem. They are typically trifoliate (Plate 2.2). However, genotypes with more than three leaflets per leaf do exist

(Ferguson and Murphy 1973). Leaflets are oblong to ovate in shape (Teuber and Brick 1988), and the middle leaflet petiolule is longer than the others (Plate 2.2).

Stems are typically square in cross section (Teuber and Brick 1988). The growing point is the meristem situated at the stem apex (Teuber and Brick 1988). Axillary nodes are present at the axil between each leaf and the stem (Teuber and Brick 1988). If the plant is grown without defoliation, branching shoots form from the lower nodes (Teuber and Brick 1988) while flowers upon racemes are borne on the upper nodes of the main shoot (Frame *et al.* 1998).

Genotypic influences over shoot morphology exist, with particular differences between genotypes grouped by winter activity rating. Decreasing winter activity is associated with a more decumbent growth habit, thinner stems and shortened internode length (Humphries and Hughes 2006). This difference in shoot morphology means that winter-dormant genotypes have a higher leaf to stem ratio than winter-active genotypes (Lodge 1986; Humphries and Hughes 2006). However, the quantitative nature of this trait means that it may be influenced by a genotype by environmental interaction. Since lucerne leaves, compared to stems, are more digestible and contain higher concentrations of protein and minerals (with the exception of potassium; Buxton *et al.* 1985; Frame *et al.* 1998), higher leaf to stem ratios and thinner stems result in increased nutritive value (Volenec *et al.* 1987).

Plate 2.2. A trifoliate lucerne leaf, showing ovate leaf shape, serrations towards the leaf apex and the middle leaflet extended on a petiolule.

2.3.1.2. Shoot growth and development

Shoots originate from two positions on the plant. Shoots that originate from buds on the crown are known as crown shoots. Shoots that arise from other shoots are referred to as axil shoots. They are derived from axil buds found at the node between leaf and stem (Plate 2.3; Leach 1968). Crown shoots exert a greater sink strength than axil shoots for plant assimilates, and they also out-compete axil shoots for light (Leach 1971; Gosse *et al*. 1988). Because of this dominance, crown shoots have greater growth rates than axil shoots (Leach 1968; Singh and Winch 1974).

As shoots develop, growth transitions from vegetative to reproductive. This process of morphological development has been summarised by Kalu and Fick (1981) and is presented in Table 2.3. Kalu and Fick (1981) extended these stages of development to give an indication of morphological development in an entire crop, using weighted averages based on mass (mean stage weight; MSW) or shoot

numbers (mean stage count; MSC). While this scale covers a broad range of plant phenological development classes it is much coarser than equivalent scales used in other crops (e.g. the cereal code system; Zadocks *et al.* 1974). As such, it is limited to predicting changes in nutritive value rather than quantitatively describing crop development stage. Changes in MSW are better than MSC in predicting changes in nutritive value (Kalu and Fick 1981).

Table 2.3. Definitions of morphological stages of development of individual lucerne shoots (Source: Kalu and Fick 1981).

Stage name	Stage no.	Stage definition
Early vegetative	0	Stem length up to 15cm; no buds, flowers or seedpods
Mid-vegetative	1	Stem length 15 to 30cm; no buds, flowers or seedpods
Late vegetative	2	Stem length greater then 30cm; no buds, flowers or seedpods
Early bud	3	1 to 2 nodes with buds; no flowers or seed pods
Late bud	4	Greater than 2 nodes with buds; no flowers or seed pods
Early flower	5	One node with one open flower (standard open); no seed pods
Late flower	6	Greater than 1 node with open flowers; no seed pods
Early seed pod	7	1 to 3 nodes with green seed pods
Late seed pod	8	Greater than 3 nodes with green seed pods
Ripe seed pod	9	Nodes with mostly brown mature seed pods

Plate 2.3. An axil shoot arising from an axially node between a leaf and the stem of a crown shoot.

Growth and development of lucerne shoots is influenced by environment factors including light, temperature and moisture availability. Floral development initiates in response to an increase in the photoperiod length, with winter-active cultivars less sensitive to changes than winter-dormant cultivars (Major *et al.* 1991). When light conditions do not trigger a transition from vegetative to reproductive growth, shoots will remain in the vegetative stages of development (Gramshaw *et al.* 1981; Sheaffer *et al.* 1988; Gramshaw *et al.* 1993).

A decrease in photoperiod length is required to initiate the physiological processes leading to cold acclimation and winter dormancy. Once initiated, decreasing temperatures are required to further the process. Cold acclimation and winter dormancy entail a partitioning of plant reserves to the perennial organs (crown and taproot) at the expense of shoot growth (Noquet *et al.* 2001; Teixeira *et al.* 2007c). However, this response is mediated by genotype, with winter-dormant genotypes partitioning greater amounts of reserves to the taproot and consequently

having a greater reduction in shoot growth than winter-active genotypes (Hodgson 1964; Teuber *et al.* 1998).

When soil moisture and light interception are not limiting, temperature is the major cause of variability in lucerne yield (Collino *et al.* 2005). Growth rates are greatest with day temperatures between 25 and 29^{o}C and night temperatures between 18 and 25^{o}C (Ueno and Smith 1970; Patterson 1993). As shoots transition from vegetative to reproductive growth, the optimum temperature for growth decreases by 5^{o}C (Fick *et al.* 1988). Excessively high temperatures (i.e. greater than 35^{o}C) reduce growth and can cause heat injury to the plant (Frame *et al.* 1998). The rate of morphological development of shoots is slowed at lower temperatures, with plants requiring an extra 3 weeks to reach flowering when grown at 17^{o}C compared to 32^{o}C (Pearson and Hunt 1972b). Leaf appearance rate and leaf area expansion rate are greatest at high temperatures and a long photoperiod and are slowest at low temperatures and a short photoperiod (Brown and Moot 2003; Brown *et al*. 2005b).

Decreasing temperature below 15^{o}C coupled with a decreasing photoperiod triggers the process that leads to winter dormancy and cold acclimation (Kanneganti *et al.* 1998a; Kanneganti *et al.* 1998b). Cold acclimation is associated with reduced shoot growth rates. However, the extent of this decrease is affected by genotype. Winter-active genotypes are not as responsive to decreasing temperatures as winter-dormant genotypes (Kanneganti *et al.* 1998a) and, as such, have greater rates of shoot elongation in autumn (Teuber *et al.* 1998). A relative comparison of autumn shoot elongation rates to well characterised check cultivars is a metric used to assign cultivars and experimental lines into winter activity classes and ratings (Teuber *et al.* 1998). The level of winter activity of a genotype also has implications for shoot growth rates in spring and summer. A positive relationship between shoot growth rate and winter activity rating has been reported in the Mediterranean climatic region of Australia (Leach 1970a; Humphries and Hughes 2006) and in North American environments (Perry and Larson 1974; Stout and Hall 1989), but not in subtropical environments (Rogers 1981; Lloyd *et al.* 1985; Lodge 1985; Lowe *et al.* 1985; Lodge 1986; Gramshaw *et al.* 1993). This highlights a genotype by environmental interaction effect on the expression of this trait and the multiple reports suggest this interaction is repeatable. The greater rates of shoot elongation during various times

of the year means that across a wide range of environments, winter-active cultivars are considered to have greater annual yields compared to winter-dormant cultivars (Leach 1970a; Stout and Hall 1989; Gramshaw *et al.* 1993; Humphries *et al.* 2006b).

Water deficits have an overriding effect on lucerne growth and development, as water is a key resource required to support metabolism (Retta and Hanks 1980; Sammis 1981). Soil water potentials below -1.0 to -1.5 MPa reduce shoot growth rates through a variety of mechanisms (Brown and Tanner 1983; Carter and Sheaffer 1983b; Grimes *et al.* 1992), which include reduced shoot elongation rates, decreased internode length, slowed leaf development rates, slowed node appearance rate, reduced leaf area expansion and reduced radiation use efficiency (Vough and Marten 1971; Carter and Sheaffer 1983b; Durand *et al.* 1989; Brown *et al.* 2009), as well as a decreasing shoot water potential (Carter *et al.* 1982; Grimes *et al.* 1992). A strong positive relationship between leaf water potential and leaf area expansion has been identified by Karmanos *et al.* (2009). Using a modelling framework, Brown *et al.* (2009) identified that out of leaf area expansion rate, node appearance rate and radiation use efficiency, leaf area expansion rate is the most sensitive to water deficits. Genotype influences the shoot water potential response to water deficit, with winter-dormant cultivars able to maintain a more favourable shoot water potential under drought conditions (Grimes *et al.* 1992). Yield under water deficit conditions in arid environments is influenced by genotype, however, rankings amongst genotypes are inconsistent between years and environments (Oloff and Hanson 2008). This signifies a potential genotype by environmental interaction typical of a complex trait. However, the inconsistency of this expression also suggests that this interaction is not repeatable. Milder environments with less exposure to abiotic stresses may allow for a more consistent genotype response.

The negative effect of water deficit on stem growth is greater than the negative effect on leaf area, leading to higher leaf to stem ratios for plants under water deficits (Carter and Sheaffer 1983b; Halim *et al.* 1989b). However, completely withholding water causes senescence of leaves on lower positions on the shoot (Brown and Tanner 1983; Halim *et al.* 1989b; Irigoyen *et al.* 1992a), reducing both the leaf to stem ratio and yield. Severe water deficit slows morphological

development by preventing the transition from vegetative growth to reproductive growth (Halim *et al.* 1989b), which, along with an increase in leaf to stem ratio, is responsible for the improved nutritive value often observed for drought stressed lucerne (Carter and Sheaffer 1983a; Halim *et al.* 1989b).

2.3.1.3. Yield components

Lucerne swards are comprised of a population of shoots and plants. The number of shoots per plant and plant density combine with shoot mass to equate to dry matter yield (Volenec 1999) as described by equation 2.1.

Equation 2.1: $\text{Yield} = \text{plant density} \times \text{shoots per plant} \times \text{mass per shoot}$

The relative contribution to yield of each yield component changes dynamically. Both the number of shoots per plant and the mass per shoot are capable of compensating for reductions in other yield components (Bolger and Meyer 1983; Volenec *et al.* 1987). The net effect of this compensation is that only minor changes in yield are observed as plant density declines (Bolger and Meyer 1983; Volenec *et al.* 1987). However, Bolger and Meyer (1983) identified that in a favourable environment the ability of individual yield components to compensate ceases if plant density declines below 19 plants/m^2. While no published information is available, the minimum number of plants required for adequate compensation in other environments is likely to be greater in less favourable environments.

Plant density in a lucerne crop continually declines as the crop ages. This decline is rapid during the first two years following establishment (Summers and Gilchrist 1991; Beuselinck *et al.* 1994) and is largely attributed to high interplant competition for moisture, nutrients and light (Beuselinck *et al.* 1994). As the crop ages, plant losses become more gradual and are the result of exposure to a combination of biotic and abiotic stress factors (Beuselinck *et al.* 1994). Due to allelopathic effects seedling recruitment is not a feasible method of increasing plant populations (Miller 1996; Jennings and Nelson 1998; 2002). Consequently the

ability to compensate for declining plant density, through increasing the number of shoots per plant, is important for the maintenance of long-term crop productivity.

The number of shoots per plant is influenced by both management and environment factors. At lower plant densities (around 43 plants/m^2), the number of shoots per plant increases with increasing light intensity (Cowett and Sprague 1963) and improvements in the phosphorus (P) and potassium (K) status of the soil (Sanderson and Jones 1993; Li *et al.* 1997). Exposure of plants to a water deficit within 14 days after defoliation leads to a reduction in number of shoots per plant, while exposure to a water deficit after 14 days of regrowth has no influence on number of shoots per plant (Brown and Tanner 1983). Increasing defoliation height increases the number of shoots produced per plant (Leach 1968; 1969; 1970b). However, this practice increases the proportion of lower yielding axial shoots in the sward, and does not increase forage yields (Volenec *et al.* 1987; Simon *et al.* 2004).

Attempts to identify what changes in yield components are responsible for changes in forage yield have been inconsistent. Increasing the mass per shoot by improving genetics (Volenec 1985), soil fertility (Li *et al.* 1998; Berg *et al.* 2005; Berg *et al.* 2007), altering defoliation timing (Teixeira *et al.* 2007a) and alleviating pest pressure (Kitchen *et al.* 1990) has been associated with increased forage yields. Yield decreases have been associated with a decline in shoot density (Undersander *et al.* 1998), and the maintenance of a high shoot density is considered important for maintaining crop productivity (Suzuki 1991). Berg *et al.* (2007) found that changes in shoot density were only associated with changes in the yield of older crops where a low plant population limited the ability for increases in mass per shoot to adequately compensate for a reduction in plant density. These contrasting results come from experiments undertaken using one cultivar (with the exception of Volenec 1985) and no replication across multiple locations. As shoot mass and shoot density are both quantitative and complex traits, differences in results highlight possible genotype by environment interaction effects on the contribution of each yield component to yield.

2.3.1.4. Photosynthesis

The photosynthetic pathway of lucerne is typical of the reductive pentose phosphate (C_3) photosynthesis pathway (Heichel *et al.* 1988) and, as such, the rate of photosynthesis is limited because of light saturation of the photosynthetic apparatus. Light saturation of individual leaves occurs at a photon flux density of 1200 to 1400 µmol/m^2/s (Sheehy and Popple 1981).

Leaves that are between 27 to 40 days old have approximately half the carbon dioxide (CO_2) exchange rate of younger (7 to 10 days old) fully expanded leaves (Pearce and Lee 1969; Ku and Hunt 1973; Hodgkinson 1974; Nicolodi *et al.* 1988). At least part of this decline can be attributed to the shading that exists within the canopy, as older leaves are capable of significantly higher rates of CO_2 exchange if shading is removed (Wolf and Blaser 1972; Meuriot *et al.* 2004a).

Air temperature also has a major effect on photosynthesis. At temperatures below 21^{o}C, net photosynthesis is reduced (Al-Hamdani and Todd 1990) and radiation use efficiency decreases (Collino *et al.* 2005). In temperate environments radiation use efficiency of lucerne is greatest during the summer months (Teixeira *et al.* 2008) which is reflective of the temperatures experienced during that time period. Increasing temperatures above 21^{o}C increases photorespiration, decreasing the efficiency of photosynthesis. For example, an increase in temperature from between 19^{o}C and 20^{o}C up to between 30^{o}C and 32^{o}C results in a 50% increase in photorespiration (Pearson and Hunt 1972a; Ku and Edwards 1977; Ku and Hunt 1977). There is limited influence from genotype on the temperature response of photosynthesis parameters (Ma *et al.* 2009).

Water deficit adversely affects photosynthesis. As leaf water potential increases, stomata close to prevent water loss through transpiration (Ottman 1999). Despite limiting the supply of CO_2, stomata closure associated with water deficit is seldom linked to a reduction in photosynthesis (Nicolodi *et al.* 1988; Irigoyen *et al.* 1992a; Bell *et al.* 2007), suggesting that other factors place limitations on photosynthesis during drought. Possible non-stomatal factors that could limit photosynthesis under drought conditions include decreases in chlorophyll content

(Zeid and Shedeed 2006), reduced efficiency of photosystems (Antolin and Sanchez-Diaz 1993; Aranjuelo *et al.* 2005) and the senescence of leaves (Brown and Tanner 1983; Halim *et al.* 1989b; Irigoyen *et al.* 1992a).

Despite the varied effects of changes in environmental conditions on photosynthesis at the individual leaf level, the main limitation to photosynthesis at the canopy level is the amount of light intercepted (Heichel *et al.* 1988). When accounting for shading within a dense canopy, light saturation of the entire canopy under favourable environmental conditions occurs at a photon flux density between 1600 and 1800 µmol/m^2/s, with a net CO_2 exchange rate of 5g CO_2/m^2/h (Gosse *et al.* 1988). This rate is reduced by environmental limitations (e.g. drought) through a reduction in leaf area rather than by a direct inhibition of the photosynthesis reactions (Erice *et al.* 2006). There is also a genotype influence on the net photosynthesis of a canopy, with winter-active cultivars having greater leaf area expansion rates and hence a greater ability to intercept light. Following defoliation this results in greater shoot growth rates of winter active cultivars compared to winter dormant cultivars (Volenec 1985).

2.3.2. The crown

2.3.2.1. Morphology

The crown is the interface between the shoots and the root system. Along with the root system it comprises the perennial portions of the plant. It has a complex branched structure originating from a single taproot and branching out into the shoots (Plate 2.4.).

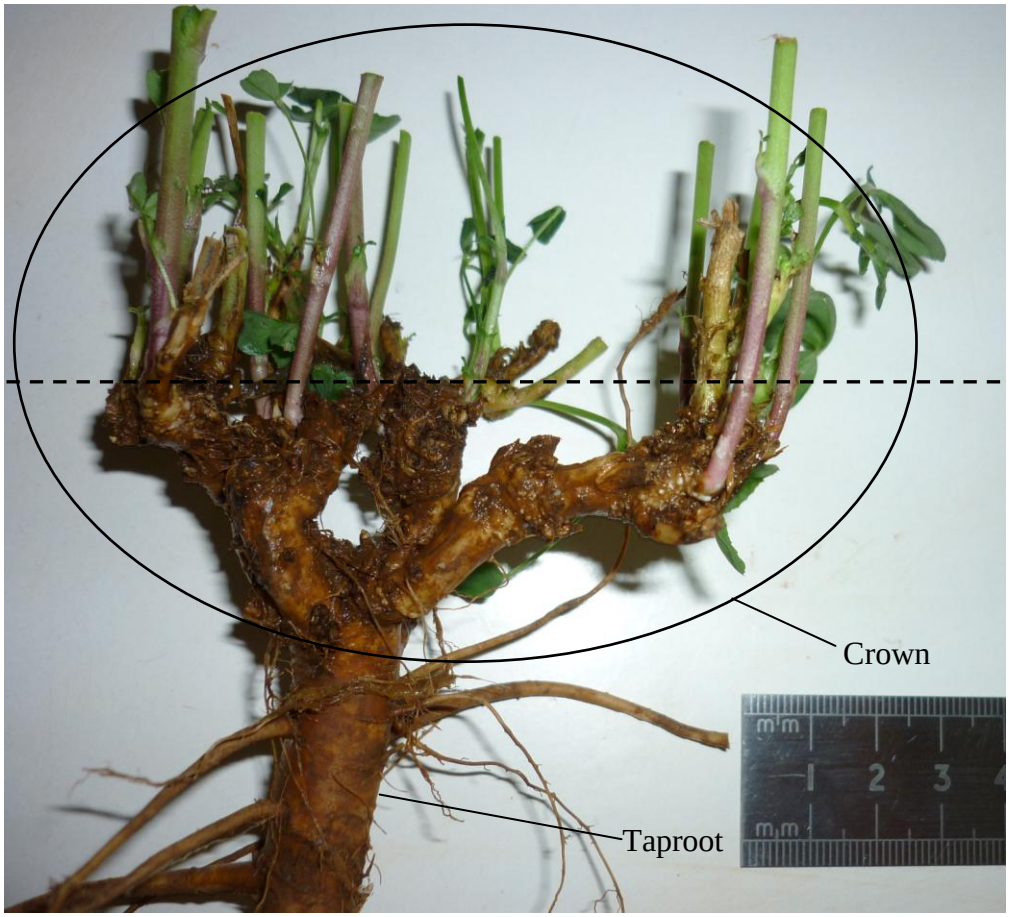

Plate 2.4. A crown from a three year old lucerne plant. The crown originates from a single taproot and branches out into shoots. The soil surface level is represented by a broken line.

Considerable variation exists in the size and shape of lucerne crowns (Brummer and Bouton 1991; Castonguay *et al.* 2006). Crown morphology is affected by genotype, with winter-dormant cultivars having broader and deeper set crowns compared to winter-active cultivars (Castonguay *et al.* 2006; Humphries *et al.* 2006b). The broader and deeper set crowns of winter-dormant cultivars are beneficial in conveying the ability to resist physical damage from freezing soils, wheel traffic, or grazing animals (Teuber and Brick 1988; Brummer and Bouton 1991; Humphries *et al.* 2006b).

Lucerne crowns develop through contractile growth of the hypocotyl of the seedling, which pulls the cotyledons below the soil surface (Perry and Larson 1974; Musgrave and Langer 1977). The cotyledonary nodes become the source of the initial crop of secondary shoots (Musgrave and Langer 1977). As the plant develops and is exposed to consecutive harvest and regrowth cycles, the crown becomes a more complex and branched structure (Plate 2.3; Teuber and Brick 1988).

Crown buds are the source of crown shoots which have a greater shoot mass compared to shoots arising from auxiliary nodes (Leach 1968; Singh and Winch 1974). Two types of buds develop and they can be distinguished by colour (Plate 2.5). Both white and green crown buds (Cunningham *et al.* 1998) can form anywhere on the crown and, as yet, no distinction in shoot morphology, growth or development has been made between the shoots which arise from green or white buds.

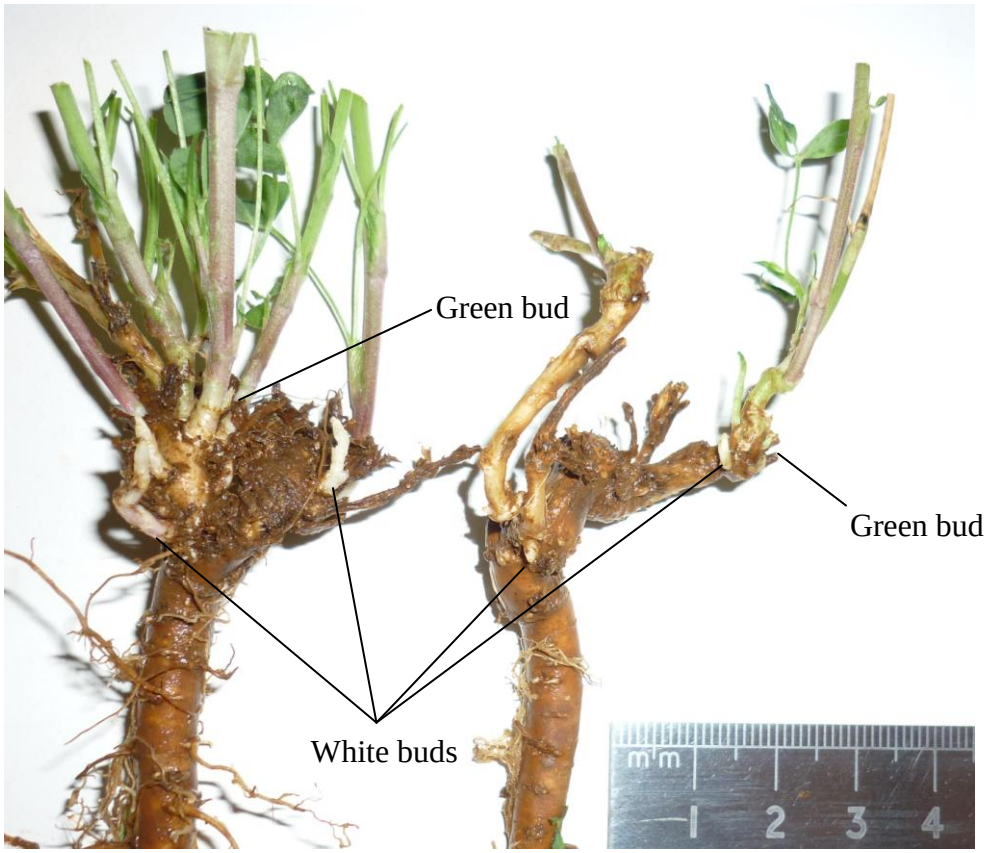

Plate 2.5. Green and white crown buds on crowns from a three year old lucerne plants.

2.3.2.2. Crown bud development

Crown buds follow a seasonal pattern in their development and elongation into shoots. Grandfield (1943) observed very few crown buds present over summer. However, the method used by this author to identify crown buds meant that no green buds were counted. Both white and green bud numbers increase through autumn and into winter (Grandfield 1943; Cunningham *et al.* 1998), until they elongate into new shoots in the following spring (Nelson and Smith 1968b). Crown bud size also increases through autumn and winter, as does the concentration of total non-structural carbohydrates (TNC) in bud tissues (Cunningham *et al.* 1998).

Water deficit has an impact on crown bud numbers in both seedlings and establishing crops. In seedlings, the number of crown buds per plant is reduced by exposure to water deficit (Cowett and Sprague 1962). Alleviating summer water deficits through irrigation increases the number of crown buds per plant present in the first winter after planting (Justes *et al.* 2002). However, no evaluation of the impact of water deficit on the number of crown buds per plant has been made for established lucerne crops.

The elongation of buds into new shoots is controlled by the plant hormones auxin and cytokine (Hall 1973; Davies 1987; Tomkins and Hall 1991). Defoliation causes a lowering in the ratio of auxins to cytokinin. This induces crown buds to elongate into new shoots and new buds to develop (Tomkins and Hall 1991). The reduction in the concentration of auxin appears to be the critical hormonal influence required to trigger crown bud elongation, as applications of exogenous cytokinin, while triggering the development of auxiliary shoots, do not cause crown buds to elongate (Tomkins and Hall 1991).

2.3.3. Taproot and root system

2.3.3.1. Morphology

The lucerne root system comprises a distinct taproot from which multiple secondary and fine roots branch (Plate 2.6; Kohl and Kolar 1976; Frame *et al.* 1998). While the taproot is capable of penetrating over 3m into the soil profile, if not inhibited by

edaphic or management constraints (McIntosh and Miller 1981; Carter and Sheaffer 1983b; Carter and Richards 2000; Humphries and Auricht 2001), the major portion of the root mass is found in the top 15 cm of the soil profile (Bennett and Doss 1960), as this zone is abundant with secondary and fine roots.

Genotypes differ in their root morphology, with winter-dormant cultivars having a greater proportion of root mass closer to the soil surface (Bennett and Doss 1960), a greater degree of root branching (Salter *et al.* 1984) and a greater total root surface area (Carter *et al.* 1982). These differences in root distribution are thought to protect the plant from physical compaction, pulling and lifting stresses (referred to as frost heaving) that are associated with freezing soils (Castonguay *et al.* 2006). They are also associated with genotypic differences in the pattern of water use and the ability to maintain greater rates of stomatal conductance under water deficit conditions (Hattendorf *et al.* 1990). These differences in root system structure would be expected to result in a repeatable genotype by environmental interaction for yield under water deficit conditions. However, Oloff and Hanson (2008) failed to observe a repeatable genotype by environmental interaction for yield with exposure to moisture stress. This suggests that other factors and plant adaptive processes also influence the performance of lucerne genotypes under dry conditions.

Plate 2.6. Lucerne taproots from the top 15 cm of the soil profile from three year old lucerne crops, showing varying degrees of branching.

2.3.3.2. Root growth and development

Root growth and development are influenced by temperature, moisture and soil structural and chemical characteristics. Root mass and degree of branching are both responsive to temperature. However, the optimal temperatures for these traits are different (Kendall *et al.* 1994). Root mass is greatest at 25^{o}C while the degree of branching is greatest at 21^{o}C (Kendall *et al.* 1994). Total root weight is reduced by water deficit (Salter *et al.* 1984). However, the proportion of fibrous roots increases when plants are exposed to a water deficit (Salter *et al.* 1984). Water logging also reduces root growth which is observed as a reduction in total root mass (Smethurst and Shabala 2003). Low soil pH (below 5.5) and toxic concentrations of aluminium both affect root growth. Aluminium toxicity causes abnormal branching and reduced secondary growth (Joost and Hoveland 1986), while soil acidity can present a barrier to root penetration (Carter and Richards 2000).

2.3.3.3. Nitrogen fixation

As a legume, atmospheric N fixation conveys a considerable competitive advantage to lucerne over grasses and non-legume weeds in N limited environments (Lutwick and Smith 1977; Nuttall *et al.* 1980). Atmospheric N fixation in legumes is achieved through a symbiotic relationship with soil-borne *Rhizobia* bacteria. For lucerne the *Rhizobia* species that forms this relationship is *Sinorhizobium meliloti* (formally *Rhizobium meliloti*; Vance *et al.* 1988; MacAdam and Nelson 2003). Symbiosis is established by the infection of the fine roots with *Rhizobia*, which leads to the formation of nodules (Plate 2.7.). These nodules provide the environmental conditions required by the *Rhizobia* to reduce atmospheric N to ammonia (MacAdam and Nelson 2003) which is made available to the plant in exchange for carbohydrates produced by photosynthesis (Vance *et al.* 1988).

Plate 2.7. Nodules on the fine roots of lucerne.

Rates of atmospheric N fixation range from 37 to 360 kg/ha/year across a broad number of environments and production systems (Heichel and Henjum 1991; Goh *et al.* 1996; Peoples *et al.* 2001). This range highlights the strong environment and management effects on N fixation. Fixation is primarily a function of biomass production (Dear *et al.* 1999; McCallum *et al.* 2000; Bowman *et al.* 2002), and as such, factors that affect shoot growth (discussed previously) limit N fixation. Some other factors, independent of shoot growth, can affect N fixation by influencing the amount of plant N derived from the atmosphere (Peoples *et al.* 1995). A high soil N status will negatively impact on the amount of atmospheric N fixed, as legumes will utilise soil mineral N in preference to N supplied by *Rhizobia* (Butler 1988; Lamb *et al.* 1995; Peoples *et al.* 1995; Armstrong *et al.* 1999). However, even at high levels of N fertilisation (840 kg/ha/year of N), lucerne will still derive 20-25% of its N requirement from the symbiotic relationship with *Rhizobia* (Lamb *et al.* 1995). Australian soils in their native state contain no *Rhizobia* species capable of forming an effective symbiosis with lucerne (Peoples and Baldock 2001). While many of the soils where lucerne is grown have been inoculated with *Rhizobia* from previous lucerne crops, seasonal variations in temperature and moisture conditions are not conducive to *Rhizobia* persisting without a host plant (Graham 1992; Triplett *et al.* 1993; Athar and Johnson 1997). As a result, the *Rhizobia* populations is often below the 100 000 cells/g of soil required for adequate nodulation (Frame *et al.* 1998). If lucerne is to be planted in paddocks where lucerne has not been grown for more than three years, inoculation of seed prior to planting is required to ensure adequate nodulation (Triplett *et al.* 1993).

2.3.3.4. Reserve storage and utilisation

Lucerne stores assimilated carbon (C) and N that are in excess of its requirements for growth (Avice *et al.* 1996a). The primary site for this storage is the taproot, and taproot reserves play an important role in plant recovery and regrowth after defoliation and in the tolerance of and recovery from biotic and abiotic stress (Volenec *et al.* 1996).

Carbon is stored in the form of non-structural carbohydrates (Habben and Volenec 1990; Dhont *et al.* 2002). Starch is the major form of carbohydrate stored (Nelson and Smith 1968a; MacAdam and Nelson 2003), with sucrose the next most abundant carbohydrate. Sucrose is stored in the cytoplasm of cells, while starch is deposited as granules within cells of bark tissue and medullar rays of the woody tissue in the taproot (Habben and Volenec 1990). Carbon translocation from shoots to roots begins after the second week of regrowth (Hodgkinson 1969; Sheehy *et al.* 1979; Habben and Volenec 1990), and continues until taproot carbohydrate levels reach a maximum at the late flower growth stage (Reynolds and Smith 1962).

Environmental conditions influence carbohydrate storage, with high temperatures decreasing carbohydrate storage (Robison and Massengale 1968; Erice *et al.* 2007), and exposure to mild water deficits increasing storage (Cohen *et al.* 1972; Erice *et al.* 2007). The environmental influences on carbohydrate storage, combined with the defoliation induced depletion and re-accumulation of taproot carbohydrate, create a seasonal pattern in C partitioning, with the amount of carbohydrate partitioned into storage increasing during autumn and winter, followed by a subsequent depletion during spring (Volenec *et al.* 1991; Castonguay *et al.* 1995; Dhont *et al.* 2002; Teixeira *et al.* 2007c).

Genotype influences the partitioning of carbohydrate into starch and sugars. Over summer, winter-dormant cultivars have greater concentrations of taproot starch compared to winter-active cultivars (Cunningham and Volenec 1998; Boschma and Williams 2008), while, over winter, taproot starch concentrations are greater in the winter-active cultivars (Cunningham and Volenec 1998). The reverse of this pattern is observed for soluble sugars, with winter-dormant cultivars having greater taproot soluble sugar concentrations over winter compared to winter-active cultivars (Cunningham and Volenec 1998; Cunningham *et al.* 1998).

Stored carbohydrates are utilised when the plant is exposed to a stress that reduces the photosynthetic capacity below which the plant requires for respiration (Avice *et al.* 1996a) and to support the plants recovery when conditions become favourable for growth to resume (Hodgkinson 1973; Avice *et al.* 1996a). Following

the removal of the photosynthetic area by defoliation, carbohydrates are utilised for respiration by the crown and roots (Khaiti and Lemaire 1992) with very little stored carbohydrate (less than 5%) being incorporated into new tissue (Avice *et al.* 1996a). Concentration and content of stored carbohydrates are both important factors in determining the overall energy status of a plant. Plants with large crowns and greater root biomass require more carbohydrates to maintain respiration immediately after defoliation.

Soil P and K status influence the utilisation of carbohydrate reserves. Plants grown under P deficient conditions, while storing greater amounts of starch, are unable to remobilise it following defoliation (Li *et al.* 1998; Berg *et al.* 2009). Potassium is required for the activation of key enzymes involved in starch synthesis (Collins and Duke 1981), and plants deficient in K have slow rates of starch accumulation (Berg *et al.* 2009). Adequate levels of soil nutrients, especially P and K, are required so that taproot carbohydrate reserves can be utilised to support plants through periods of stress.

Despite its apparent importance in stress tolerance and regrowth after defoliation, there is conflicting information as to the effect of TNC reserve levels on the rate of regrowth and stress tolerance. Some reports identify correlations between TNC storage or rapid TNC depletion either with associated high rates of regrowth (Graber *et al.* 1927; Habben and Volenec 1991; Avice *et al.* 1997a; Dhont *et al.* 2002) or increased plant survival during exposure to stress (Grandfield 1943; Dhont *et al.* 2004; Boschma and Williams 2008). Others have either not identified any relationship between the two (Volenec 1985; Fankhauser and Volenec 1989; Boyce and Volenec 1992; Avice *et al.* 1997b; Teixeira *et al.* 2007b), or have identified that N reserves have an equally important relationship (Dhont *et al.* 2004). These discrepancies have led to the causality in the relationship between carbohydrate reserves and stress tolerance to be questioned (Volenec *et al.* 1996).

Taproot N reserves show a pattern of depletion and re-accumulation following defoliation (Hendershot and Volenec 1993; Avice *et al.* 1997b). Following defoliation, 95% of taproot N reserves are translocated to sites of regrowth

where they are incorporated into newly growing shoots (Avice *et al.* 1996a). Using a modelling approach, Lemaire *et al.* (1992) showed that one third of nitrogen in the herbage at 50 to 60 days after defoliation is sourced from stored taproot nitrogen. Immediately following defoliation, these reserves must supply almost all of the N for the formation of new tissue, as N fixation is reduced by 88% within 24 hours of defoliation and does not recover to rates capable of supplying sufficient N for growth until after two weeks of regrowth (Vance *et al.* 1979; Vance and Heichel 1981; Kim *et al.* 1993). In addition, uptake of soil mineral N is limited during early regrowth (Vance and Heichel 1981). The importance of stored N to supply the plant's N requirements for the formation of new tissue, post-defoliation, is highlighted by a strong positive correlation between pre-defoliation taproot N reserves and the rate of subsequent regrowth (Avice *et al.* 1997b; Justes *et al.* 2002; Dhont *et al.* 2006b). Given the role N reserves in regrowth, the total amount of N reserves rather than their concentration is a better indicator the plants N reserve status.

Nitrogen is stored in the form of soluble proteins and amino acids (Ta *et al.* 1990; Avice *et al.* 1997b; Meuriot *et al.* 2004a; Dhont *et al.* 2006b), with soluble protein the primary form of stored N (Hendershot and Volenec 1993; Avice *et al.* 1997b). In the soluble protein pool, Cunningham and Volenec (1996) have characterised three highly abundant proteins (comprising 20% of the soluble protein pool) that exhibit a cyclic behaviour of preferential depletion and accumulation through a regrowth cycle (Barber *et al.* 1996), and are characteristic of vegetative storage proteins (VSPs; Staswick 1994). Vegetative storage proteins accumulate within cell vacuoles associated with starch granules in the ray parenchyma cells of the taproot (Avice *et al.* 1996b). The predicted amino acid sequence of the largest (32 Kd) of these proteins has 78%, 75% and 68% identity to the predicted amino acid sequence of a type three chitinase from *Medicago truncatula* Gaertn. (Volenec *et al.* 2002), *Sesbania rostrata* Scop. and Soy bean (*Glycine max* L. Merr; Meuriot *et al.* 2004b) respectively. The two smaller (15 and 19 kD) VSPs have not yet been identified. β-amylase has been proposed as a fourth VSP in lucerne. It is highly abundant (up to 10% of the soluble protein pool), exhibits a cyclic pattern in depletion and re-accumulation associated with defoliation, and does not appear to

have a role in starch degradation following defoliation (Boyce and Volenec 1992; Gana *et al.* 1998).

Environmental conditions influence the partitioning of N into storage. A shortening in photoperiod (Noquet *et al.* 2001) increases storage of N as soluble proteins and amino acids, and increases the abundance of the VSP in the soluble protein pool. While low temperatures have no effect on N storage (Noquet *et al.* 2001), raising temperatures from 28^{o}C to 32^{o}C decreases the amount of N partitioned into VSPs (Erice *et al.* 2007). Phosphorus and K deficiency inhibit the utilisation and re-accumulation of VSPs during the regrowth of lucence and will delay the expression of genes encoding for β-amylase and the high molecular weight VSP (Berg *et al.* 2009).

Reports on the impact of water deficits on N storage have been inconsistent. Under field conditions, irrigation of lucerne during establishment increases the amount of soluble protein and the abundance of VSP in the taproot. However, no evaluation of established crops was undertaken (Justes *et al.* 2002). In controlled conditions, fully-grown plants exposed to a water deficit store greater amounts of N compared to fully watered plants (Erice *et al.* 2007). This conflict remains to be resolved, and may reflect differences in genotypes, management and environment.

Stress signalling in lucerne may affect the accumulation of VSPs. Applications of methyl jasmonate (a plant developmental and stress signalling substance) increase VSPs accumulation in lucerne taproots (Meuriot *et al.* 2004b). This finding, along with the increase in abundance of VSP associated with drought observed by Erice *et al.* (2007) and the identification of the high molecular weight VSP as a chitinase (Meuriot *et al.* 2004b), has led these investigators to suggest that VSPs may also have secondary roles associated with stress tolerance. However, in lucerne, no definitive physiological role, other than N storage has been identified for these proteins.

The impact of temperature, photoperiod and soil water availability on the levels of taproot N reserves means that partitioning N into storage follows a seasonal pattern similar to that of taproot TNC levels, with an increase in taproot N levels

though autumn and into winter (Wilding *et al.* 1960; Hendershot and Volenec 1992; Li *et al.* 1996; Cunningham and Volenec 1998; Cunningham *et al.* 1998; Dhont *et al.* 2006b; Teixeira *et al.* 2007c), followed by a decrease in spring and summer (Hendershot and Volenec 1992; Li *et al.* 1996; Cunningham and Volenec 1998; Teixeira *et al.* 2007c).

2.3.3.5. Winter dormancy and cold acclimation

Winter dormancy and cold acclimation encompass the processes that allow lucerne to adapt to subzero temperatures and ensure the survival of the taproot and crown over the winters experienced in the northern latitudes of North America and Europe (Castonguay *et al.* 2006). While such extreme winters are not experienced in the cool temperate region of Australia, autumn temperatures and changes in day length are adequate to trigger the process of cold acclimation. As such, the changes in taproot reserve accumulation and composition, and the slowing in shoot elongation associated with cold acclimation have an impact on seasonal growth patterns.

Cold acclimation begins in autumn with a shortening in the photoperiod (Hodgson 1964). The specific rate of change in photoperiod appears to be the critical factor for triggering the cold acclimation response rather than a specific photoperiod *per se* (Castonguay *et al.* 2006). For example, despite other environmental conditions being appropriate, the rapid shortening in photoperiod experienced in the extreme northern latitudes (e.g. Alaska) does not trigger cold acclimation in otherwise freezing tolerant cultivars developed for more southern latitudes (e.g. continental USA; Hodgson 1964; Bula *et al.* 1965; Klebesadel 1971).

Once the cold acclimation process is initiated by a shortening photoperiod, decreasing temperatures are required to advance it. Cold acclimation begins as air temperatures decrease below 15^{o}C (Kanneganti *et al.* 1998a; Kanneganti *et al.* 1998b), proceeds as temperatures decrease below 10^{o}C, and accelerates as temperatures decrease towards 5^{o}C (Bula *et al.* 1965; McKenzie and McLean 1980; Paquin and Pelletier 1980). The process concludes when temperatures reach between 0 and -2^{o}C, at which point maximum freezing tolerance and dormancy are

attained (Paquin and Pelletier 1980). The cold acclimation process can be interrupted and reversed if temperatures increase above 10^{o}C for several days (Paquin and Pelletier 1980). Defoliation in autumn prior to the plants attaining full dormancy (Belanger *et al.* 1992; Haagenson *et al.* 2003a), irrigation during summer and autumn (Heinrichs 1973), and waterlogged conditions during autumn (McKenzie *et al.* 1988) reduce the level of cold tolerance attained.

The cold acclimation response is genotype specific. Winter-active cultivars do not initiate the process as early as winter-dormant cultivars (Bula *et al.* 1965; Shih *et al.* 1967; Paquin and Pelletier 1980) and do not attain the same level of freezing tolerance (Stout and Hall 1989; Sheaffer *et al.* 1992; Cunningham and Volenec 1998; Cunningham *et al.* 1998; Cunningham *et al.* 2003; Haagenson *et al.* 2003a; Dhont *et al.* 2006b). Consequently, persistence of winter-active cultivars is limited in environments that expose the plant to freezing conditions over winter (Sheaffer *et al.* 1992; Cunningham *et al.* 2001), and winter activity rating (Teuber *et al.* 1998) has been used as a useful predictor for winter survival of genotypes and cultivars (Sheaffer *et al.* 1992). However, several lucerne genotypes have been identified as having a greater level of freezing tolerance than what their classified winter activity rating would suggest (Brouwer *et al.* 2000; Brummer *et al.* 2000), indicating that the genetic linkage between winter dormancy and freezing tolerance can stretched (Castonguay *et al.* 2006). However, as yet these genotypes have not been incorporated into a named cultivar.

Cold acclimation results in changes in plant physiology, taproot carbohydrate and N reserve pools as well as the expression of specific genes. Winter dormancy is initiated by a decreasing photoperiod. Once initiated the concentration of both phytochrome B and abscisic acid in the herbage increases. However, this response is mediated by genotype with concentrations of both phytochrome B and abscisic acid greater in more winter dormant genotypes (Wang *et al.* 2008). With the onset of winter dormancy, DM portioning to the roots is increased while, root respiration rates are reduced to one seventh of what they are during summer (Teixeira *et al.* 2008; 2009). Taproot starch concentration and content decrease with an associated increase in the size of the soluble sugar pool (Cunningham and Volenec 1998;

Cunningham *et al.* 1998; Dhont *et al.* 2004). The composition of the soluble sugar pool changes with an increase in raffinose, stachyose and sucrose concentration (Castonguay *et al.* 1995; Cunningham *et al.* 2003) and a decrease in the concentration of glucose and fructose (Castonguay *et al.* 1995). The concentration of raffinose and stachyose in the taproots is correlated with the plant's freezing tolerance (Castonguay *et al.* 1995; Cunningham *et al.* 2003).

Like sugars, specific amino acids and proteins accumulate during the process of cold acclimation. The concentrations of proline (Paquin and Pelletier 1981; Dhont *et al.* 2006b), arginine and histidine (Dhont *et al.* 2006b), as well as total amino acids (Dhont *et al.* 2004), increase during cold acclimation. The accumulation of VSPs (Noquet *et al.* 2001; Dhont *et al.* 2006b), β-amylase (Gana *et al.* 1998) and other as yet unidentified polypeptides (Cunningham *et al.* 1998) in the taproot soluble protein pool coincides with cold acclimation. Despite accumulation and changes in composition of taproot N pools, there appears to be no relationship under field conditions, between the concentration or abundance of specific N reserves and freezing tolerance (Dhont *et al.* 2006b). However, spring growth is correlated with the amount of taproot N reserves stored over winter (Justes *et al.* 2002; Dhont *et al.* 2003; Dhont *et al.* 2006b; Teixeira *et al.* 2007c).

Extensive study of the molecular mechanisms of cold acclimation in lucerne has identified many genes that appear to regulate the acquisition of freezing tolerance (Castonguay *et al.* 2006). These genes have been grouped into families of cold acclimation responsive (CAR), cold acclimation specific (CAS) and cold-regulated (COR) genes. There is great diversity in the function of the punitive proteins these genes encode for, with predicted amino acid sequence homology to nuclear signalling proteins (Monroy *et al.* 1993; Haagenson *et al.* 2003a), dehydrins (Wolfraim and Dhindsa 1993; Wolfraim *et al.* 1993; Remus-borel *et al.* 2010) and raffinose family carbohydrate synthesis (Cunningham *et al.* 2003). These proteins would protect taproot and crown cells from freezing damage by maintaining the integrity of membranes, preventing damage from reactive oxygen species, and by increasing the solute concentration in the cell to reduce the freezing point (Castonguay *et al.* 2006). Several authors (Cunningham *et al.* 2001; Cunningham *et*

al. 2003; Haagenson *et al.* 2003a; Dhont *et al.* 2006a) have noted a positive association between the expression of these genes and surviving freezing stress. However, in some cases, an increase in transcript abundance was not associated with survival, bringing into question the cause-effect relationship between some of these genes and freezing tolerance (Haagenson *et al.* 2003a).

It is clear that genotype and environment conditions influence the physiological processes and plant morphology that underpin the growth and development of lucerne, both separately and through potential genotype by environment interactions. In the case of yield components, and also in the role of stored carbohydrate and N in determining regrowth potential and stress tolerance, conflicting information from previously published research is evidence of possible genotype by environment interactions on the expression of these traits. This possibility is further strengthened by the complex and quantitative nature of these traits. An assessment of the possible genotype by environmental interactions to determine their extent and repeatability is required to enable the selection of appropriate lucerne cultivars for the southern dairy regions of Australia. This can only be achieved through a detailed understanding of how genotype and environment interact to affect lucerne morphology and physiology in a cool temperate climate.

2.4. Genotype by environment interaction effects on yield and persistence

2.4.1. Natural environment

The natural environment encompasses factors beyond management control and includes the climatic and edaphic conditions to which the plant is exposed. The only economically viable method available to address limitations presented by the natural environment is to exploit repeatable genotype by environment interactions either through the selection of cultivars adapted to a particular environment or the development of better adapted cultivars through plant breeding (Cooper *et al.* 2006).

Evidence of a genotype by environment interaction on growth can be found in the differences between shoot growth rates of winter-active cultivars compared to winter-dormant cultivars in North American climates (Perry and Larson 1974; Stout and Hall 1989) and in some locations and years in Mediterranean climate regions of Australia (Leach 1970a; Humphries and Hughes 2006), but not in the subtropical regions of Australia (Rogers 1981; Lloyd *et al.* 1985; Lodge 1985; Lowe *et al.* 1985; Lodge 1986; Gramshaw *et al.* 1993). The cool temperate regions of Australia are intermediate climate types, with less extreme winters than those experienced in North America, and cooler summers than those experienced in Mediterranean environments. Shoot growth of lucerne genotypes with differing levels of winter activity remains to be defined for this environment.

An interaction between genotype and photoperiod affects the process of cold acclimation, and hence winter survival in environments that expose the plant to subzero temperatures over winter. This genotype by photoperiod interaction effect on cold acclimation and winter survival has been observed by Hodgson (1964), Bula *et al.* (1965), Klebesadel (1971) and Heinrichs (1973) when comparing the performance of freezing tolerant cultivars in subarctic Alaska and Canada to their performance in the continental USA. When reviewing these experiments, Castonguay *et al.* (2006) concluded that cultivars to which ssp. *sativa* was the major contributor of parent material, with minimal contribution from ssp. *falcata*, were insensitive to the rapid decrease in photoperiod experienced during autumn in the northern latitudes. Consequently they did not initiate the process of cold acclimation as they would typically do when grown in continental USA. The southern dairy regions of Australia are situated at an equivalent latitude to continental USA and have similar changes in photoperiod. Photoperiod changes should be able to be sensed by cultivars comprised of ssp. *sativa*, with no need to rely on ssp. *falcata*.

The genotype by temperature interaction is highlighted by the distribution of genotypes across the North American continent. The freezing temperatures experienced in the northern regions prevent the use of winter-active cultivars (Melton *et al.* 1988) as they do not possess the capacity to adapt to freezing winter temperatures (Stout and Hall 1989; Sheaffer *et al.* 1992; Cunningham *et al.* 2001).

The range of winter activity levels that are successfully grown increases as winters become milder. In areas with minimal risk of experiencing subzero temperatures during winter, winter-active cultivars are favoured (Melton *et al.* 1988).

The cool temperate dairy regions of Australia have considerably milder winters compared to areas of equivalent latitudes in the northern hemisphere, with daily average minimum temperatures remaining above freezing (Figure 2.1). There should be no limitations to the use of cultivars with any level of winter activity, as there is no need for adaption to freezing conditions. However, anecdotal reports suggests that winter-dormant cultivars are the best adapted genotypes in these regions (Knox *et al.* 2006). It is possible that winter stress may not be the limiting environmental factor preventing the successful use of winter-active material in cool temperate environments. This has been proposed by Berg *et al.* (2007), who identified that, in an environment that exposed plants to freezing winters, the majority of plant death occurs over the summer period. Clearly there is a need to scientifically evaluate the yield and persistence of a range of winter-activity genotypes in the cool temperate regions of Australia to determine their suitability for use in these environments.

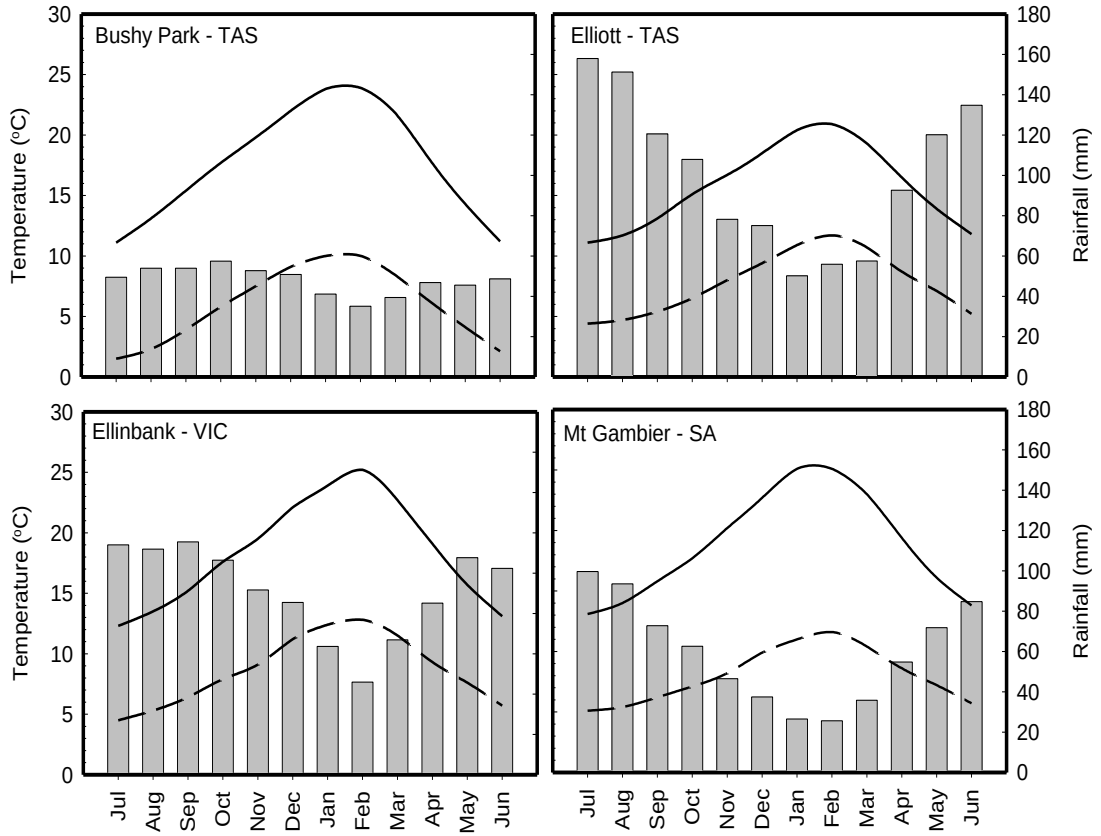

Figure 2.1. Average monthly maximum (solid lines) and minimum temperatures (broken lines) and total monthly rainfall (bars) for four locations in the temperate dairy regions of Australia (Bushy Park, Tasmania; Elliott, Tasmania; Ellinbank, Victoria; Mt Gambier, South Australia). Data sourced from the Australian Bureau of Meteorology (2009).

2.4.2. Management environment

Management encompasses factors that can be controlled or influenced (e.g. defoliation, irrigation and soil fertility). Cultivar recommendations from subtropical regions of Australia suggest that winter-dormant cultivars are best adapted to extensive pastoral systems with low levels of management inputs, while winter-active cultivars are suitable for intensive irrigated hay production and grazing enterprises (Lloyd *et al.* 2002). This recommendation highlights that, when selecting

a lucerne cultivar, consideration of genotype by management interactions is just as important as consideration of the genotype by natural environment interactions.

2.4.2.1. Defoliation

Defoliation management and genotype interact to affect yield and persistence of lucerne. There is a difference in the performance of lucerne genotypes under continuous grazing compared to mechanical harvesting or rotational grazing (Kaehne *et al.* 1993; Bouton and Gates 2003). Under optimal conditions, there is little genotype by defoliation interaction on the performance of genotypes when rotationally grazed compared to mechanically harvested (Lodge 1985; Bouton and Gates 2003). This suggests that genotypes differ in their ability to tolerate stress specific to continuous grazing. Stresses specific to continuous grazing include continual trampling, tugging, waste excretion and repeated defoliation of regrowing shoots (Smith *et al.* 1989). A consistent finding when genotype performance is evaluated under continuous grazing is that winter-dormant genotypes are better adapted to continuous grazing than winter-active genotypes (Smith *et al.* 1989; Brummer and Bouton 1991; Kaehne *et al.* 1993; Smith and Bouton 1993; Bouton and Gates 2003; Humphries *et al.* 2006b). This adaptation is attributed to the broader and deeper set crowns of winter-dormant genotypes compared to winter-active genotypes (Brummer and Bouton 1991; Humphries *et al.* 2006b). Dairy farms in the temperate regions of Australia employ rotational grazing as a matter of course. As such, the full range of winter activity genotypes should be able to be grown successfully, as no genotype by defoliation method interaction is observed when comparing mechanically harvested and rotationally grazed crops (Lodge 1985; Bouton and Gates 2003). The genotype by defoliation method interaction would need to be considered if the intention was to continuously graze the crop, as winter-dormant genotypes are best suited to this purpose.

The faster regrowth rates of winter-active cultivars compared to winter-dormant cultivars (Leach 1970a; Perry and Larson 1974; Stout and Hall 1989; Humphries and Hughes 2006) and the lower sensitivity to environmental triggers that

initiate reproductive growth (Major *et al.* 1991) and cold acclimation (Bula *et al.* 1965; Shih *et al.* 1967; Paquin and Pelletier 1980; Hendrickson *et al.* 2008), would suggest that the timing of defoliations need to be adjusted with respect to the genotype's level of winter activity. However, Gramshaw *et al*. (1993) showed that the defoliation interval that maximised yield was the same for a range of genotypes, and that defoliations timed to coincide with crown bud elongation were appropriate for a broad range of winter activity ratings under a range of environmental conditions. Timing defoliations to coincide with crown bud elongation ensures that crown buds are readily available for the formation of new shoots and that taproot reserve levels are adequate to support regrowth (Lowe *et al.* 2002).

Late autumn and winter management is critical for maintaining productive lucerne crops in a wide range of environments (Tomes *et al.* 1972). Defoliation during cold acclimation interrupts the acclimation process, alters the composition of taproot reserve pools and increases the risk of injury when exposed to freezing temperatures (Belanger *et al.* 1992; Haagenson *et al.* 2003a). However, autumn defoliation does not have the same level of impact on freezing injury as does the level of winter activity (Belanger *et al.* 1992; Haagenson *et al.* 2003a). When evaluating the impact of autumn defoliation on the abundance of gene transcripts associated with cold acclimation, Haagenson *et al.* (2003a) found that defoliation had little impact on their expression, or in the case of a winter-active cultivar, the abundance of transcripts actually increased with defoliation. These counter intuitive results led these authors to question the cause-effect relationship between the abundance of transcripts for some cold acclimation genes and freezing tolerance (Belanger *et al.* 1992; Haagenson *et al.* 2003a). In climates that do not experience freezing winters, late autumn and winter defoliation may be beneficial to lucerne production and persistence, by reducing pest and disease pressure on the plants in this period (Pelton *et al.* 1988; Wynn-Williams *et al.* 1991; Bell and Guerrero 1997; Moot *et al.* 2003; Chocarro *et al.* 2005). These evaluations have been undertaken across a range of climate types from cool temperate New Zealand to semi-arid California, with genotypes that ranged in winter activity classifications of semi winter-dormant to highly winter-active. This consistency in results suggests that in

areas not exposed to freezing winter temperatures, all genotypes can be grazed over winter and that this practice improves lucerne production. In environments where lucerne becomes dormant, winter defoliations should be timed to occur when plant growth is slowest (Collins and Taylor 1980; Douglas 1986). This reduces shoot growth during the remainder of the winter period and ensures that adequate taproot reserves are available to support rapid growth when temperatures increase in spring (Collins and Taylor 1980; Douglas 1986).

2.4.2.2. Irrigation and water deficits

Evaluations of the effects of genotype by water deficit on lucerne performance have either identified no interaction effects on yield (Carter *et al.* 1982; Hattendorf *et al.* 1990; Grimes *et al.* 1992), or, when an interaction existed, it was not repeatable between years and locations (Oloff and Hanson 2008). This is counter intuitive as winter-dormant cultivars are capable of maintaining a greater shoot water potential under drought conditions (Carter *et al.* 1982; Grimes *et al.* 1992) and have a greater degree of root branching (Bennett and Doss 1960; McIntosh and Miller 1981), which would suggest winter-dormant genotypes would perform better than winter-active genotypes under conditions of limited water. Evaluations of genotype by irrigation effects on yield have been undertaken under glasshouse conditions (Carter *et al.* 1982), or in North American environments with exposure to freezing winters (Retta and Hanks 1980; Hattendorf *et al.* 1990), or in semi-arid climates (Undersander 1987; Grimes *et al.* 1992). No evaluation has been made in milder cool temperate environments, which may allow potential differences between genotypes to be more readily and consistently expressed.

Direct evidence of a genotype by irrigation interaction effect on yield components has been identified in glasshouse experiments by Perry and Larson (1974). While winter-active genotypes had the greatest number of shoots per plant when plants were fully watered, when plants were exposed to a water deficit, winter-dormant cultivars maintained a greater number of shoots per plant. When growing under a water deficit, winter-dormant genotypes also produce a greater number of

leaves per shoot compared to winter-active genotypes (Perry and Larson 1974). These responses suggest that genotypes with contrasting levels of winter dormancy may utilise different strategies to adapt to water deficits. Identifying how genotypes differ in adaption to water limited conditions will be important in the development of genotype specific best management practices.

While the influence of genotype on taproot carbohydrate and N reserve pools has been documented (Avice *et al.* 1997b; Kalengamaliro *et al.* 1997; Cunningham and Volenec 1998; Boschma and Williams 2008) and considerable effects of irrigation/water deficit on these reserve pools observed (Cohen *et al.* 1972; Justes *et al.* 2002; Erice *et al.* 2007), no evaluation of the effect of irrigation on taproot reserve pools in genotypes of contrasting levels of winter activity has been made. Evidence of possible genotype by water deficit interaction effects on taproot reserves is provided by the contradicting results of Justes *et al.* (2002) and Erice *et al.* (2007), where these authors observed contrasting effects of water deficit on VSP abundance. As taproot reserves are important in stress tolerance and support plant recovery after the removal of stress, the extent of genotype influence of carbohydrate and N reserves during water deficit will need to be considered when developing best management practices for managing lucerne through periods of low water availability.

Irrigation of lucerne in summer and autumn reduces the freezing tolerance attained in winter (Heinrichs 1973). This is attributed to the extra growth achieved during the irrigation period reducing taproot reserve pools to levels that are not adequate to support the process of cold acclimation (Heinrichs 1973). While the studies undertaken by Heinrichs (1973) on cold acclimation and winter survival evaluated a diverse range of winter activity genotypes under irrigated and dryland conditions, experimental design limitations prevented the comparisons of winter survival amongst diverse genotypes. The possible genotype by irrigation interaction on taproot reserve pools may also affect the process of cold acclimation and subsequent winter dormancy.

2.4.2.3. Soil fertility

Adequate levels of mineral nutrition are critical for reserve storage and remobilisation (Collins and Duke 1981; Li *et al.* 1998; Berg *et al.* 2009) and genotypes differ in their seasonal partitioning of carbohydrate and N into storage (Avice *et al.* 1997b; Kalengamaliro *et al.* 1997; Cunningham and Volenec 1998; Boschma and Williams 2008). However, there appears to be no consistent genotype by soil fertility interaction effect on production, persistence and physiological responses to environmental stimuli (Tindall and Hurst 1988; Gossen *et al.* 1994; Lloveras *et al.* 2001). All genotypes respond positively to increasing soil mineral concentrations into the ranges considered adequate to support high levels of production, with improvements in production, persistence and nutritive value (Tindall and Hurst 1988; Gossen *et al.* 1994; Lloveras *et al.* 2001; Turan *et al.* 2008; Lissbrant *et al.* 2009).

To ensure maximum plant production with minimal fertiliser wastage, best management practice is to use soil testing and analysis of results to guide decisions about fertiliser applications. The depth of soil testing should reflect the depth of soil in which the majority of the fine root mass is present (i.e. 7.5 to 10cm for temperate pastures and 10 to 30cm for various field crops; Brown 1993). Genotype differences in root system anatomy (Bennett and Doss 1960; Carter *et al.* 1982; Salter *et al.* 1984) mean that soil testing for the winter-active genotypes may need to be to a deeper depth than for winter-dormant genotypes. However, as the majority of fine roots for all genotypes are present in the upper 15 cm of the soil profile (Bennett and Doss 1960), soil testing to this depth would ensure that fertiliser decisions are made using information reflecting nutrient availability to the plant. Recent investigations have highlighted possible nutrient interactions between magnesium and potassium and phosphorus and potassium. This demonstrates the importance of considering the overall nutrient status of the soil when making fertiliser decisions (Lissbrant *et al.* 2010).

2.5. Identifying and managing lucerne genotypes for the dairy industry in the cool temperate regions of Australia

This review suggests that genotype by environment and genotype by management interactions are likely to affect the performance of lucerne within the cool temperate dairy regions of Australia for complex and quantitative traits such as yield, persistence and quality. While interactions between genotypes and various aspects of defoliation management are well understood, and there is not likely to be a major interaction between genotypes and soil fertility status, the impact on key growth processes of genotype by irrigation and genotype by environment interactions are not well understood.

There is a need to quantify the extent and repeatability of genotype by environment interactions in cool temperate climates and the effect of these interactions on agronomic and physiological traits. This will ensure that appropriate cultivars can be selected and will lay the foundation for the development of genotype-specific best management practices for lucerne grown in cool temperate environments. Chapters 3 to 8 of this thesis will describe a series of experiments to test the following hypotheses:

1. Genotype by environment interaction effects are present for lucerne grown in cool temperate climates;
2. Genotype by environment interaction effects will influence yield, persistence, yield components and nutritive value parameters;
3. Water deficit will be a major environment factor determining genotype by environment interactions; and
4. Water deficit and genotype will interact to influence the key physiological processes of canopy development, reserve storage and cold acclimation and winter dormancy.

CHAPTER 3

Genotype by environment interactions of lucerne in a cool temperate climate

3.1. Introduction

The genetic diversity of lucerne has lead to the development of a broad range of cultivars suited to particular environments and farming systems (Quiros and Bauchan 1988). This specific adaptation is observed as genotype by environment interactions. While the adaptation of lucerne in regions of Australia has been explored using biophysical and logic modelling approaches (Hill 1996; Robertson 2006), these assessments provides no information regarding the adaptability of lucerne genotypes with contrasting levels of winter dormancy to the differing environments and hence provided no indication of the presence and extent of genotype by environment interactions.

There is a paucity of information available on the performance of lucerne genotypes in cool temperate climates. Cool temperate climates are an intermediate climatic type with less extreme winters than those experienced in the continental climatic zone of North America and cooler summer than those of Mediterranean climates. Anecdotal evidence suggests that the persistence of winter-active lucerne genotypes is poor in cool temperate climates and, as such, these genotypes are not suitable for utilisation as part of the dairy feedbase in those regions. However, substantial variations exist in the climatic and edaphic conditions across the cool temperate dairy regions of Australia. This chapter will examine the hypothesis that genotype by environment interactions are present for the production and persistence of lucerne genotypes of differing winter activities in cool temperate climates. The objective was to analyse data from two field experiments investigating the performance of lucerne cultivars and experimental lines using winter activity class as the experimental factor to determine the existence and extent of genotype by environment interactions in a cool temperate climate.

3.2. Materials and methods

3.2.1. Experimental locations and design

Two locations in Tasmania, Forth (41°12'00" South, 146°16'12" East, red ferrosol soil, Isbell 2002; Humic Etrodox soil, Soil Survey Staff 1990) and Cranbrook (42°00'00" South, 148°01'48" East, red ferrosol soil, Isbell 2002; Humic Etrodox soil, Soil Survey Staff 1990) were used as experimental sites. The experiments were both irrigated and lasted for a period of three years from sowing in January/February 1989. At each location four replications of 7.5 m × 1.5 m plots were laid out in randomised complete blocks.

3.2.2. Planting

Planting occurred at Cranbrook on 2 January 1989 and at Forth on 16 February 1989. Seeds were drilled into a fully prepared seedbed on 150 mm row spacing, at a rate of 10 kg/ha, and to a depth of 15 mm. Seed was freshly inoculated with *Rhizobia* and lime coated prior to planting. Before planting, 51 kg/ha of K and 18 kg/ha of P were broadcast in the form of muriate of potash (50% K) and molybdenum (Mo) fortified single superphosphate (9% P 0.025% Mo).

3.2.3. Crop management

Fertilisers were applied once a year in early May at Cranbrook and twice a year in late March and early September at Forth. For each application at Cranbrook, 51 kg/ha of K and 18 kg/ha of P were applied, while at each application at Forth 42.5 kg/ha of K and 15 kg/ha of P were applied. All fertilisers were applied in the form of muriate of potash and molybdenum fortified single superphosphate.

Volumetric soil water content (SWC) was maintained between 100% and 75% of the drained upper limit (DUL) by sprinkler irrigation, as determined by estimated evapo-transpiration (ET) calculated using the method described by Doorenbos and Pruitt (1977). Sprinklers were arranged in a diamond pattern to ensure even irrigation applications.

Defoliations were timed to coincide with crown bud elongation. At Cranbrook, plots were grazed to 50 mm residual height with sheep, with a grazing

duration of no longer than five days. As no sheep were available at Forth, plots were defoliated to 50 mm with a reciprocating mower and the cut material removed from the plots. These defoliation practices reflect current best management practice for the defoliation of lucerne in Australia (Lowe *et al.* 2002).

In order to reduce weed density Velpar DF (hexazinone, 750 g/kg) was applied annually in mid-September at a rate of 3 L/ha.

The lower fertiliser applications and the higher summer temperatures combined to create a low yield potential environment at Cranbrook, while, the milder summer temperatures and higher fertiliser applications at Forth created a high yield potential environment.

3.2.4. Cultivars and experimental lines

A total of 39 and 36 commercially-available cultivars and experimental breeder lines from the Australian lucerne breeding program were evaluated at Cranbrook and Forth, respectively. Of these, 36 were common to both locations. Estimated winter activity rating of each of the experimental lines was acquired from the South Australian Research and Development Institute (SARDI) database, which collates results from the SARDI lucerne breeding field evaluation program. Winter activity rating was used to group each entry into four winter activity classes; dormant (3.0-4.4), semi-dormant (4.5-5.9), winter-active (6.0-7.9), and highly winter-active (8.0-9.0). Characteristics of the commercially available cultivars used at both locations are given in Table 3.1. The total number of lines and cultivars in each winter activity class were seven dormant, nine semi dormant, 13 winter-active and seven highly winter-active at Forth and eight dormant, 11 semi dormant, 13 winter-active and seven highly winter-active at Cranbrook.

3.2.5. Measurements

Seedling counts were undertaken when the first true (unifoliate) leaf emerged (10 February 1989 at Cranbrook and 15 March 1989 at Forth). Live seedlings were counted along randomly selected 250 mm lengths of rows, and seedling density (plants/m^2) was calculated using the row spacing.

Prior to defoliation, three 0.25 m^2 quadrats were defoliated to 50 mm height using hand shears. The cut material was gathered and weighed. Moisture content was determined by drying a sub-sample of the collected material at 100^oC in a forced-air oven until a constant weight was achieved. Dry matter (DM) yield was calculated using the moisture content.

Plant frequency was determined in the winter prior to the completion of each experiment (4 April 1991 at Forth and 26 June 1991 at Cranbrook) using the 100 square method described by Lodge and Gleeson (1984). This entailed the placement of a 1m by 1m quadrate at a fixed location within the plot. The quadrate was divided into 100 10 by 10cm squares. The number of squares were a lucerne plant was present was counted and the percentage of the squares containing a lucerne plant was calculated as plant frequency.

Plots were regularly scouted for pest damage and disease incidence. When pest damage or disease incidence was observed, plots were visually scored from zero to five, with a score of five assigned to the most damaged plot and a score of zero assigned to the least damaged plot in the experiment.

Table 3.1. Winter activity class and disease and pest susceptibility ratings (NR: no rating available; Su: susceptible; MR: moderately resistant; R: resistant; HR: highly resistant) of released lucerne cultivars used in the Forth and Cranbrook experiments.

Cultivar	Winter activity class	Spotted alfalfa aphid	Blue-green aphid	Pea aphid	Phytophthora root rot	Leptosphaerulina leaf disease	Reference
DuPuits	Dormant	NR	NR	NR	NR	NR	Wiersma and Hartman (2000)
Pioneer 532	Dormant	MR	NR	NR	MR	NR	Wiersma and Hartman (2000)
Pioneer 5432	Dormant	HR	NR	R	MR	NR	Wiersma and Hartman (2000)
Pioneer 545	Dormant	MR	Su	Su	MR	NR	Wiersma and Hartman (2000)
WL 320	Dormant	R	MR	MR	R	NR	Wiersma and Hartman (2000)
WL Southern Special	Semi winter-dormant	R	NR	R	MR	NR	Wiersma and Hartman (2000)
Aurora	Winter -active	HR	MR	MR	R	LR	Lloyd *et al.* (2002)
Pioneer 581	Winter-active	HR	LR	LR	MR	NR	Wiersma and Hartman (2000)
Pioneer 5929	Highly winter-active	R	HR	R	R	NR	Wiersma and Hartman (2000)
WL 516	Highly winter-active	HR	HR	HR	HR	NR	Wiersma and Hartman (2000)
WL 605	Highly winter-active	HR	HR	HR	HR	NR	Wiersma and Hartman (2000)

3.2.6. Statistical analysis

Dry matter yield of all entries at each location was analysed as a blocked one way unbalanced analysis of variance (ANOVA) with winter activity class as the factor. Differences between winter activity classes were identified using Fisher's least significant difference (LSD). Unless otherwise stated, significance was accepted when $P < 0.05$.

Modified joint linear regression analysis (Digby 1979) was undertaken on total annual yields and total yields in each season (summer, autumn, winter and spring) for each winter activity class and entry. A similar application of this technique has been used to evaluate the sensitivity to environment of annual ryegrass cultivars and experimental lines (annual and spring yields), and perennial ryegrass cultivars and experimental lines (annual and total yields from three years), in the subtropics (Lowe *et al.* 2007; 2008), as well as, the uptake of cadmium in potatoes grown across Australia (McLaughlin *et al.* 1994). Environment in this analysis was defined as each year-location combination (six in total). Any entry that appeared at only one location was not included in the analysis. The sensitivity of each entry and winter activity class was plotted against the final estimates of yield. As proposed by Finlay and Wilkinson (1963), entries with a sensitivity around 1.0 show little difference in their responses to environmental conditions from that of the average response of the species to environment (i.e. minimal genotype by environment interaction). Entries with sensitivities above or below 1.0 exhibit variability in their response to environmental conditions compared to the average of the species (i.e. genotype by environment interactions exist). Entries with minimal genotype by environment interaction and high mean yield should be reliable over a range of environmental conditions. A sensitivity below 1.0 suggests specific adaptation to low yield potential environments while sensitivity above 1.0 suggests specific adaptation to high yield potential environments (Finlay and Wilkinson 1963). Modified Joint linear regression analysis and ANOVA were undertaken using Genstat 11th edition (VSN International Ltd, Hemel Hempstead, UK).

The impact of winter activity class on disease incidence and pest damage scores was determined with Friedman's test using SAS 9.1 (Proc FREQ, SAS Institute, Cary, NC, USA)

3.2.7. Climatic conditions

Climate data at Forth were collected from the Australian Bureau of Meteorology weather station on site. Climate data for Cranbrook was generated as a patched point data set (Jeffrey *et al.* 2001) with rainfall data collected at the Australian Bureau of Meteorology rainman station on site. A summary of the data from each location is given in Figures 3.1 and 3.2.

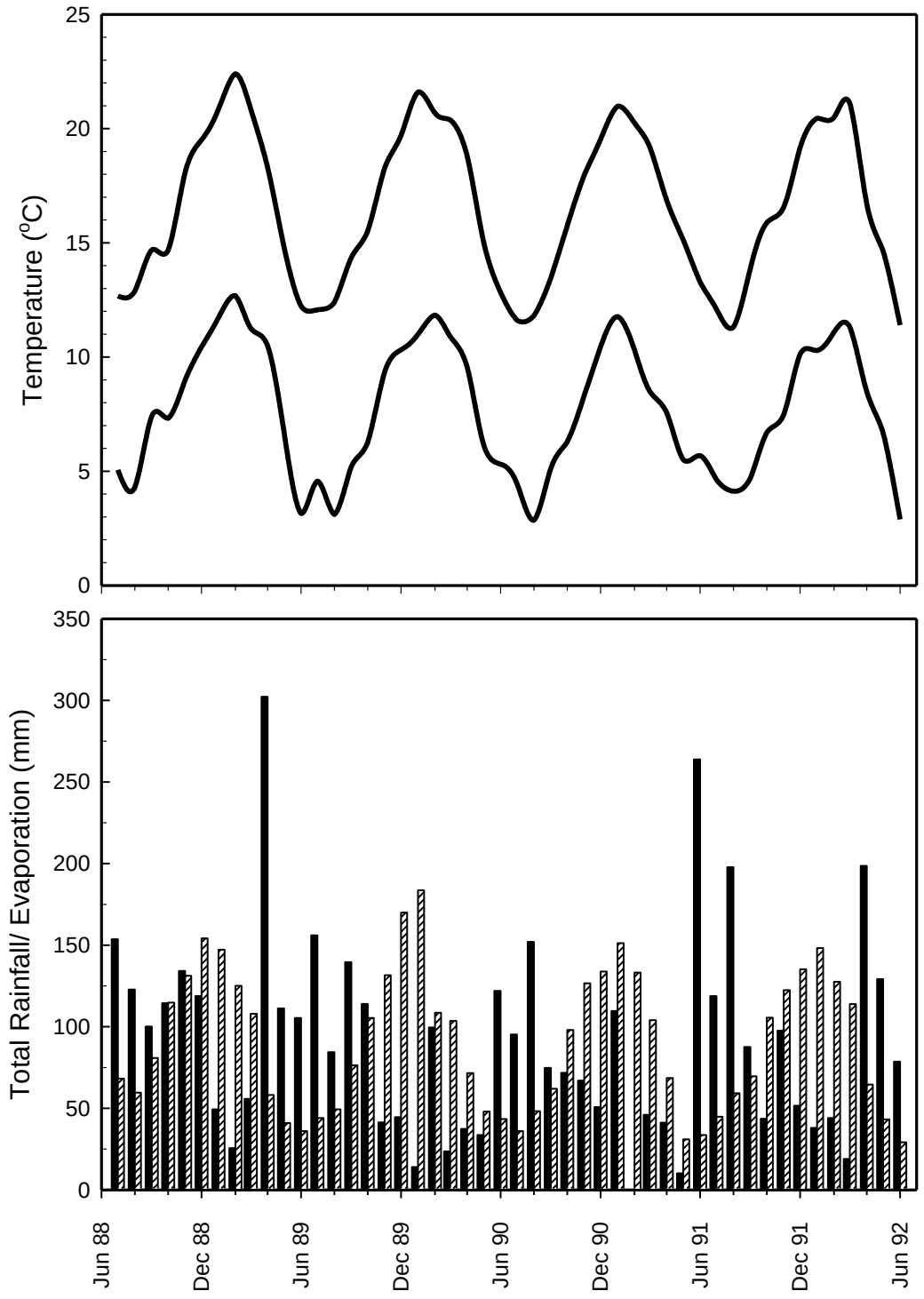

Figure 3.1. Average daily maximum (solid line) and minimum temperatures (broken line) and the total monthly rainfall (solid bars) and evaporation (hatched bars) between June 1988 and June 1992 at Forth, Tasmania.

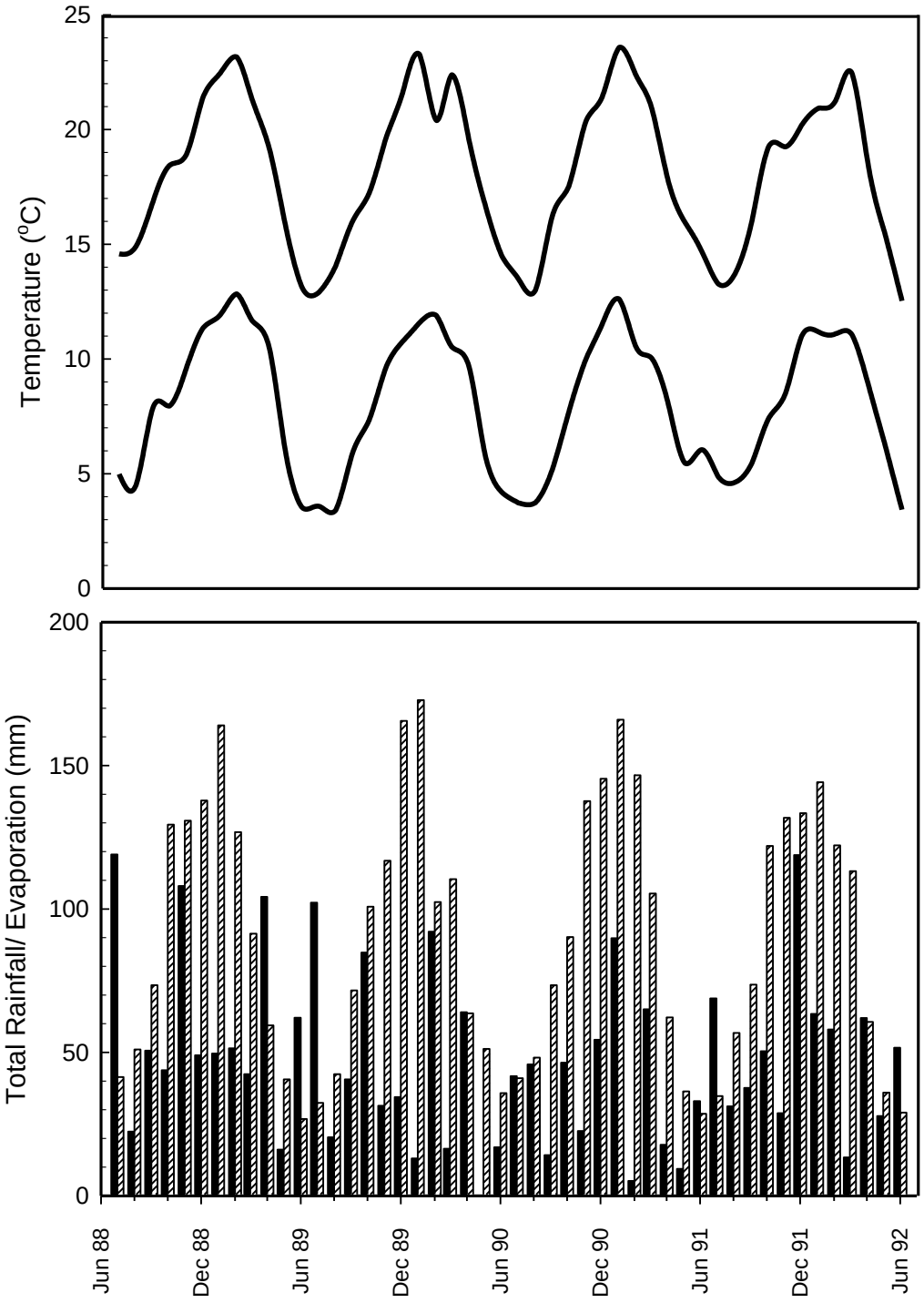

Figure 3.2. Average daily maximum (solid line) and minimum temperatures (broken line) and the total monthly rainfall (solid bars) and evaporation (hatched bars) between June 1988 and June 1992 at Cranbrook, Tasmania.

3.3. Results

3.3.1. Total and seasonal dry matter yields

At Forth, highly winter-active genotypes yielded the greatest total DM for the three years of production and were significantly different from the winter-dormant genotypes (Table 3.2). The opposite trend was observed at Cranbrook, where the highly winter-active genotype produced significantly less DM than the other genotypes (Table 3.2). An extra three harvests were undertaken at Forth compared to Cranbrook. These harvests occurred during the late winter of 1990 (one harvest) and the winter and spring of 1991 (two harvests).

Differences in seasonal DM yields between winter activity classes at Cranbrook were first evident after the first winter of the experiment (1989). At Forth however, it was not until after the second winter (1990) that total seasonal DM yield differences between winter activity classes became apparent (Table 3.2). As these differences developed, they reflected the same response as for total DM yield at each location, with winter-active genotypes producing more DM at Forth and less DM at Cranbrook.

3.3.2. Dry matter yield at each harvest

There was little difference in DM yield between the winter activity classes in the first six harvests at Forth, with the only significant difference occurring in the second harvest where winter-dormant genotypes out-yielded the highly winter-active genotypes (Table 3.3). This is in contrast to the results from Cranbrook, where in three out of the first six harvests, winter activity class influenced yield (Table 3.3). In the second harvest at Cranbrook, both the winter-dormant and highly winter-active genotypes yielded more DM than the other winter activity classes. In the following harvests, yields of each winter activity genotype were significantly different, with the winter-dormant genotypes yielding the highest. In the winter of 1990 this pattern was reversed at Cranbrook.

At Forth in 1990/91 and 1991/92 there were significant increases in yield with increasing winter activity in all except three harvests. This was not observed at Cranbrook. In harvests at Cranbrook where significant yield differences between the winter activity types occurred, the winter-dormant genotypes yielded more than the winter-active genotypes. These harvests occurred in late spring and early summer in 1990 and mid-summer in 1992.

3.3.3. Plant persistence

There was no effect of winter activity class on the number of plants established at either location (Table 3.4). Plant frequency counts after 2.5 years of production at Forth did not reveal any influence of winter activity class on plant frequency. This is in contrast to the results from Cranbrook, where after 2.5 years of production the highly winter-active genotypes had a lower plant frequency than the other winter activity genotypes (Table 3.4).

3.3.4. Pest damage and leaf disease incidence

Insect pests damage and leaf disease incidence were observed in the Forth experiment on 19 October 1989 and in the Cranbrook experiment on 16 October 1989. There was no effect of winter activity class on the score for leaf disease incidence at Forth or Cranbrook (median scores of 2 in both locations), or of pest damage at Cranbrook (median score of 2). There was a significant effect ($P < 0.01$) of winter activity rating on the scores for pest damage at Forth where the median score for the winter dormant class was 1 compared to a median score of 2 for each of the other genotypes.

Table 3.2. Impact of winter activity class on the total and annual dry matter production (kg/ha) of lucerne over four years at two locations, Forth (sown 16/02/89) and Cranbrook (sown 02/01/89) in Tasmania.

Location	Genotype	Total yield	1st year (Sowing to 31/06/89)	2nd year (01/7/89 to 31/6/90)	3rd year (01/07/90 to 31/06/91)	4th year (31/06/91 to 05/02/92)
		Total yield per year (kg DM/ha)				
Forth	Number of harvests	16	1	5	6	4
	Dormant	58562	3429	22770	21573	11470
	Semi dormant	59910	3548	21462	23845	12325
	Winter-active	60666	3431	21863	24037	12715
	Highly winter-active	61665	3277	21380	26108	12379
	LSD (P = 0.05)	1938	ns†	ns	1067	696
Cranbrook	Number of harvests	13	1	5	5	2
	Dormant	38154	3511	15894	12789	5960
	Semi dormant	38102	3312	15349	12919	6523
	Winter-active	37322	3375	15184	12534	6229
	Highly winter-active	34380	3226	14544	11433	5178
	LSD (P = 0.05)	1665	ns	588	884	918

ns†, no significant effect

Table 3.3. Impact of winter activity class on the dry matter yields (kg/ha) of each harvest of lucerne over four years at two locations, Forth (sown 16/02/89) and Cranbrook (sown 02/01/89) in Tasmania.

				Forth				
Harvest number	1	2	3	4	5	6	7	8
Harvest date	13/03/89	03/11/89	06/12/89	16/01/90	26/02/90	08/05/90	01/08/90	31/10/90
Winter activity class				Yield (kg DM/ha)				
Dormant	3429	4482	5614	5037	4983	2651	1006	5257
Semi dormant	3548	3954	5401	4634	4873	2597	1622	5763
Winter-active	3431	4015	5535	4909	4713	2688	1796	5789
Highly winter-active	3277	3679	5312	4974	4830	2786	2102	6147
LSD (P = 0.05)	ns[†]	369	ns	ns	ns	ns	161	538
Harvest number	9	10	11	12	13	14	15	16
Harvest date	12/12/90	14/01/91	19/02/91	18/04/91	03//07/91	13/11/91	16/12/91	21/01/92
Winter activity class				Yield (kg DM/ha)				
Dormant	5410	3992	3228	2680	680	3372	3900	3518
Semi dormant	5592	4119	3417	3333	1270	3515	3828	3712
Winter-active	5545	4336	3357	3214	1380	3469	3902	3964
Highly winter-active	5988	4680	3664	3528	1480	3221	3727	3951
LSD (P = 0.05)	ns	310	237	300	146	ns	Ns	362
				Cranbrook				
Harvest number	1	2	3	4	5	6	7	8
Harvest date	09/02/89	23/10/89	12/12/89	23/01/90	13/03/90	21/06/90	14/11/90	19/12/90
Winter activity class				Yield (kg DM/ha)				
Dormant	3511	3542	4324	4034	2818	1176	4611	2965
Semi dormant	3312	3067	4144	3892	2892	1354	4553	2782
Winter-active	3375	2985	3989	3935	2883	1391	4465	2696
Highly winter-active	3226	3480	3830	3926	2784	1524	3699	2461
LSD (P = 0.05)	ns	212	294	ns	ns	138	453	235
Harvest number	9	10	11	12	13			
Harvest date	21/01/91	12/03/91	26/06/91	10/12/91	04/02/92			
Winter activity class				Yield (kg DM/ha)				
Dormant	2632	2030	551	2389	3571			
Semi dormant	2636	2272	677	2610	3913			
Winter-active	2452	2192	729	2351	2378			
Highly winter-active	2426	2133	714	2204	2974			
LSD (P = 0.05)	ns	ns	ns	ns	645			

ns[†], no significant effect

Table 3.4. Lucerne seedling density counts (plants/m^2) at establishment and plant frequency counts (%) after 2.5 years of production at Forth and Cranbrook, Tasmania.

Winter activity	Dormant	Semi winter-dormant	Winter-active	Highly winter-active
Location	Seedling density at establishment (plants/m^2)			
Forth	207	203	208	194
LSD (P = 0.05)			ns†	
Cranbrook	202	220	223	214
LSD (P = 0.05)			Ns	
Location	Plant frequency counts (%) after 2.5 years			
Forth	70	72	70	69
LSD (P = 0.05)			Ns	
Cranbrook	41	42	38	31
LSD (P = 0.05)			6	

ns†, no significant effect

3.3.5. Sensitivity of genotype to environment

Sensitivity calculated from annual yields (Figure 3.3) shows that winter-active genotypes are relatively insensitive and well adapted to all environmental conditions. Semi winter-dormant genotypes showed sensitivity close to that of the winter-active genotypes but were slightly more adapted to low yield potential environments. The winter-dormant genotypes showed the best adaptability to low yield potential environments. The plants of the highly winter-active genotypes showed the most sensitivity to environmental conditions and were adapted to high yield potential environments. The sensitivities of the individual cultivars and experimental lines were loosely grouped around the average for that winter activity class. However, considerable variation was present for sensitivity and adaptability.

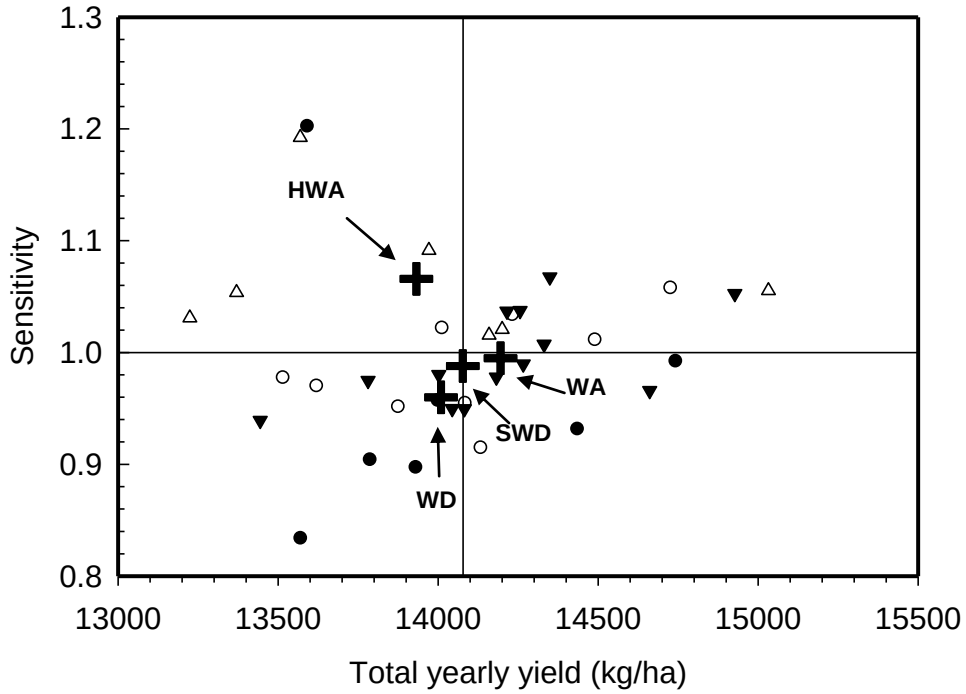

Figure 3.3. The relationship between sensitivity to Cranbrook and Forth environments in Tasmania and total annual yields of winter-dormant (WD; ●), semi winter-dormant (SWD; ○), winter-active (WA; ▼) and highly winter-active (HWA; △) lucerne cultivars and experimental breeder's lines. Average sensitivity and yield of each winter activity class (+) are also presented.

The sensitivity of each winter activity class to environment changed throughout the year (Figure 3.4). Highly winter-active and winter-dormant genotypes showed the greatest changes throughout the year. The highly winter-active genotypes were adapted to high yield potential environments through spring and summer. However, in autumn and winter, the yield of this group was still greater than the average performance of all types under all environmental variation. The winter-dormant genotypes showed adaptability to low yield potential environments in autumn, winter and spring. In spring, the yield of the winter-dormant genotypes was greater than the average performance of all winter activity classes. The DM yield of the semi winter-dormant genotypes and the winter-active

genotypes remained relatively stable throughout the year. The greatest genotype by environment interaction effect on yield was observed in winter for all winter activity classes, while genotype by environment interaction for yield was lowest in summer.

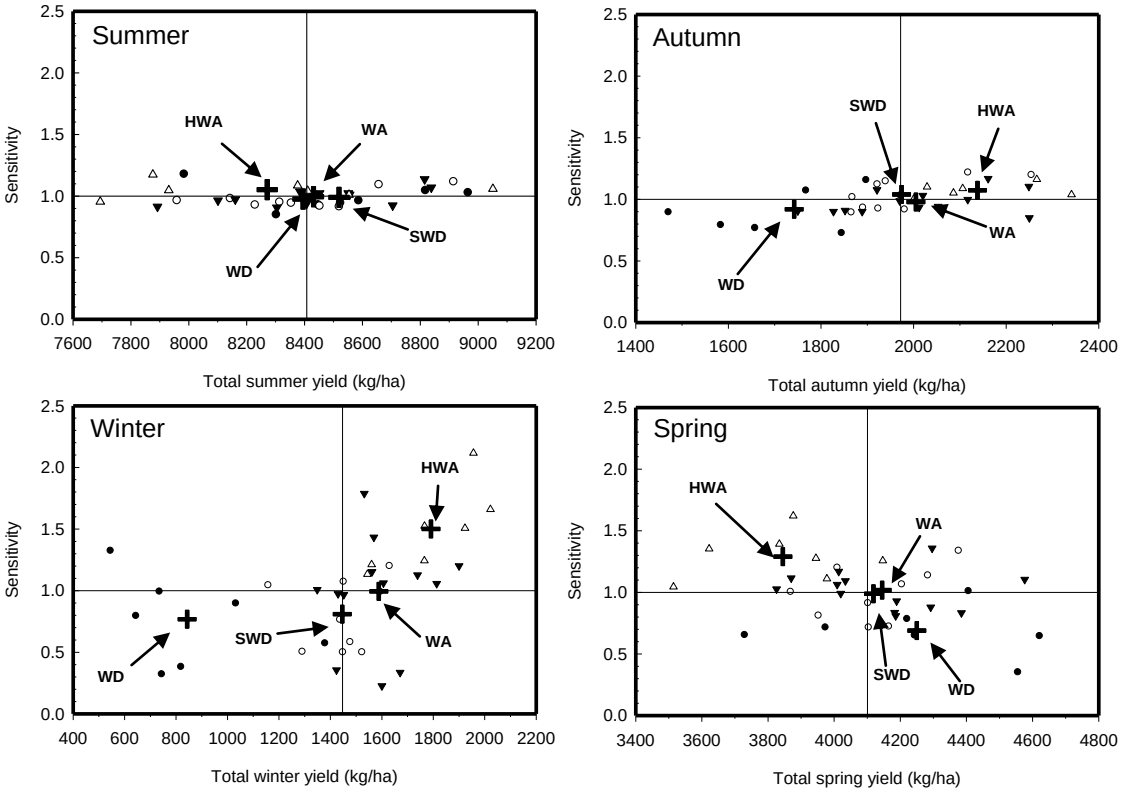

Figure 3.4. The relationship between sensitivity to Cranbrook and Forth environments in Tasmania and total summer, autumn, winter and spring yields of winter-dormant (WD; ●), semi winter-dormant (SWD; ○), winter-active (WA; ▼) and highly winter-active (HWA; △) lucerne cultivars and experimental breeder's lines. Average sensitivity and yields of each winter activity class (+) are also presented.

3.4. Discussion

This study has shown that natural and management environment variation across the pastoral regions of cool temperate Tasmania affects the relative production and persistence of lucerne genotypes. Highly winter-active genotypes where the greatest yielding at Forth and winter-dormant genotypes the greatest yielding at Cranbrook. Winter-active cultivars have greater rates of shoot elongation in Mediterranean type climates in Australia and in North American environments (Hendershot and Volenec 1989; Stout and Hall 1989; Humphries and Hughes 2006). Greater rates of shoot elongation led to the higher DM yields of highly winter-active genotypes at Forth. At Cranbrook, lower DM yields of the highly winter-active genotypes were due to poorer persistence of these plants, compared to other genotypes. Pest and disease pressure is considered one of the main causes of stand decline in lucerne crops(Lowe *et al.* 1985; Lowe *et al.* 1987; Summers and Gilchrist 1991). Observable pest damage and disease incidence only occurred once at each location and as such, had minimal impact on total production in both experiments. Only at Forth was a significant effect from winter activity rating on pest damage observed. As with leaf disease incidence at this location, and with both pest damage and leaf disease incidence at Cranbrook, median scores were on the lower end of the rating scale. As such, overall limitations on production caused by these biotic factors would have been minimal.

The sensitivity analysis indicates that highly winter active cultivars were specifically adapted to high yield potential environments while winter dormant cultivars were the best adapted genotype to low yield potential environments. However, considerable variation was observed between individual entries within a winter activity class. Previous assessments have highlighted that considerable genetic variability is present within lucerne cultivars and experimental lines for complex traits such as yield and nutritive value (Julier *et al.* 2000). The large variability within a winter activity class highlights the potential to develop cultivars that are able to adapt to more or less favourable environmental conditions within any level of winter production.

In the current evaluation, the specific sensitivity of the winter activity classes to environment became apparent in winter and spring, when cool temperatures would have limited growth. Increased sensitivity also could be expected in late autumn. However, in these experiments, all except one of the autumn harvests occurred early in autumn prior to cool temperatures limiting growth.

Highly winter-active genotypes at the lower yield potential environment of Cranbrook (lower fertiliser inputs and higher summer temperatures compared to Forth) recorded the lowest persistence of the winter activity classes. While persistence of winter-active types of lucerne is problematic under continuous grazing (Smith *et al.* 1989; Humphries *et al.* 2006b) there is no difference in persistence between winter activity classes when rotationally grazed (Lodge 1985). Winter is considered the critical period of the year when the majority of plant losses in otherwise healthy lucerne crops occur (Frame *et al.* 1998). In contrast to this, a recent study has observed that the period of plant death in the temperate regions of North America occurred over summer, following exposure of plants to freezing conditions over winter (Berg *et al.* 2005). In the current study, plants at both locations were exposed to similar winter temperatures (average maximums of 13.6 and 12.5^{o}C and average minimums of 4.2 and 4.2^{o}C at Cranbrook and Forth respectively), but warmer summer temperatures were experienced at Cranbrook (average maximums of 21.8 and 20.0^{o}C and average minimums of 11.5 and 10.7^{o}C at Cranbrook and Forth respectively). It is possible that, in both the study by Berg *et al.* (2005) and in the current study at the Cranbrook site, warmer temperatures over spring/summer promoted plant growth and imposed significant inter-plant competition on damaged plants, preventing them from recovering from winter stress. This would have left the plants weakened and unable to adapt to the summer grazing pressure experienced at this location, or to disease and insect attack. Further to this, the experiment at Cranbrook was not as intensively managed as the experiment at Forth, as highlighted by the lower and less frequent applications of fertilisers and the less intensive defoliation management (grazing vs. cutting). This reduced

management may have exacerbated the loss of plants in the highly winter-active class and reduced DM yield of all cultivars and experimental lines.

The results of the sensitivity analysis used in the current study reflects the anecdotal recommendations matching winter activity rating to farming systems in subtropical Australia. Winter-active lucerne cultivars are recommended for high input hay production systems (i.e. high yield potential environments), whereas winter-dormant cultivars are recommended for extensive pastoral systems (i.e. less favourable environments; Lloyd *et al.* 2002; Knox *et al.* 2006). These recommendations reflect the sensitivity of the winter activity classes identified in the current study, with winter-dormant genotypes being better suited to less favourable environments and winter-active genotypes able to best exploit more favourable environments.

Production from temperate forage species in the cool temperate regions is limited in summer by warm temperatures and low rainfall, and in winter by cold temperatures (Rawnsley *et al.* 2007). Lucerne would have greater summer production potential than the current predominantly perennial ryegrass based pastures, as maximum growth of lucerne is achieved at temperatures between 25 and 29^{o}C (Ueno and Smith 1970; Patterson 1993) compared with 18^{o}C for ryegrass (Kemp *et al.* 1999). Furthermore the deep-rooted nature and drought tolerance of lucerne would convey an advantage under dryland conditions. While no direct evidence regarding the performance of differing winter activity classes under dryland conditions is available from the current study, the adaptation to less favourable environments demonstrated by the winter-dormant genotypes suggests that these genotypes are best suited to dryland grazing. However, cold temperatures would still limit forage production over winter. In intensively irrigated forage production systems, where highly winter-active genotypes of lucerne would be able to best exploit the favourable summer growth conditions, minimal sacrifices to winter forage production would be expected, compared to perennial ryegrass.

The study described in this chapter has shown that genotype by environment interactions on yield and persistence exists for irrigated lucerne grown in cool

temperate climates and that the expression of this interaction is most apparent in winter and spring. This has confirmed the complex and quantitative nature of these traits and has shown that their expression is strongly influenced by genotype by environmental interactions. Further studies are required to confirm that this interaction is repeatable in a predictable manner before it can be considered when making cultivar selections. Furthermore the current experiment investigated the performance of lucerne genotypes in a high and low yield potential environment under irrigation. Given the potential and current use of lucerne under dryland conditions, research into the expression of genotype by environment interactions under dryland conditions is required.

CHAPTER 4

Yield, yield components and shoot morphology of four contrasting lucerne cultivars grown in three cool temperate environments

4.1. Introduction

The assessment of genotype by environment interactions made in Chapter 3 and previously published assessments from Australian environments (Michaud *et al.* 1961; Lloyd *et al.* 1985; Lowe *et al.* 1985; Lodge 1986) have been based on DM yield and persistence. These assessments provide no information on the role or relative contribution of yield components in determining yield of contrasting genotypes. A greater understanding of the genotype by environmental interactions affecting yield may be attained by investigating the simpler yield component traits that contribute to yield. Similarly, an understanding of how plant morphology (namely morphological development and leaf to stem ratio) are influenced by genotype by environmental interactions may provide insight into potential genotype by environmental interactions for forage nutritive value. Unfortunately, previously published assessments of genotype, environment and crop management effects on yield components and nutritive value have been undertaken in environments where growing seasons are limited by freezing temperatures and snow cover (Miller *et al.* 1969; Volenec *et al.* 1987; Sanderson and Jones 1993; Berg *et al.* 2005; 2007). As yet no assessment of these traits or the genotype by environmental interactions affecting them has been undertaken in cool temperate environments.

This chapter will examine the hypothesis that genotype by environment interactions influence the relative contribution of each yield component to total DM yield, while genotype will exhibit strong control over nutritive value traits across a range of environments. To further build on the findings presented in Chapter 3, dryland and irrigated environments will be examined.

4.2. Materials and methods

4.2.1. Site descriptions

Experiments were undertaken at two locations, Elliott (41°4'48" South, 145°46'12" East, 1200 mm annual rainfall, red ferrosol; (Isbell 2002; Humic Eutrodox soil, Soil Survey Staff 1990) and Cambridge (42°49'48" South, 147°30'00"East, 500 mm annual rainfall, brown sodosol; (Isbell 2002; Udic Haplustalfs soil, Soil Survey Staff 1990) in Tasmania, Australia. The soil chemical and physical properties to a depth of 450 mm at each environment prior to applications of fertilisers and lime and the commencement of the experiment are outlined in Table 4.1.

Table 4.1. Field texture, Olsen extractable phosphorus (P), Colwell extractable potassium (K), KCl-40 extractable sulphur (S), $pH_{(CaCl)}$ and $pH_{(water)}$ of soil at Cambridge and Elliott in Tasmania to a depth of 450 mm prior to the addition of fertiliser and lime.

Depth (mm)	Field texture	Phosphorus (mg kg^{-1})	Potassium (mg kg^{-1})	Sulphur (mg kg^{-1})	$pH_{(CaCl)}$	$pH_{(water)}$
			Cambridge			
0 - 75	Loamy sand	35.7	176.0	6.7	6.0	6.0
75 - 150	Loamy sand	24.3	65.0	7.2	5.6	6.1
150 - 450	Clay loamy sand	9.9	84.0	10.0	4.7	5.7
			Elliott			
0 - 75	Light clay	27.3	230.5	38.7	5.3	6.4
75 - 150	Light clay	12.9	108.5	22.6	5.4	6.1
150 - 450	Light medium clay	5.8	68.3	41.0	4.8	5.2

Weather data (temperature, rainfall, wind speed and solar radiation) at Elliott were collected by the Australian Bureau of Meteorology weather station on site. At Cambridge, weather data were collected at 10-minute intervals with a HOBO weather station and data logger (Onset Computer Corporation, Bourne, MA, USA). Estimated ET was calculated from observed data as described by Allen *et al.* (1998).

Climatic variables at each location are presented in Figure 4.1. At both experimental sites, crops were grown under dryland conditions. The experiment was duplicated at Elliott under irrigation, creating a third environment.

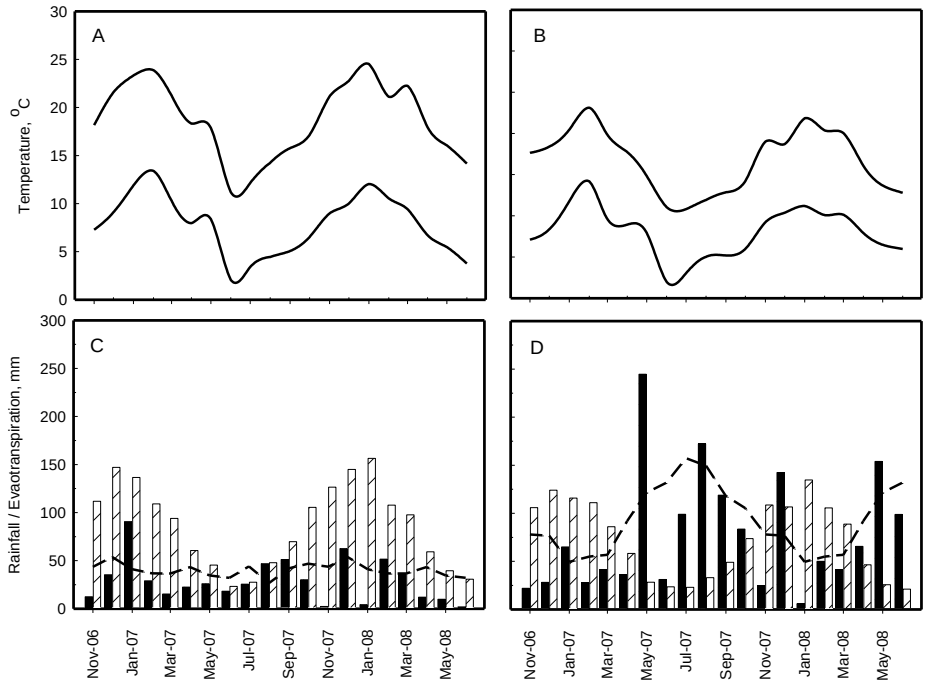

Figure 4.1. Mean daily maximum and minimum temperatures for each month (solid lines) at Cambridge (A) and Elliott (B), and total monthly rainfall (solid bars), estimated evapo-transpiration (hatched bars) and long term average rainfall (broken line) for Cambridge (C) and Elliott (D), from November 2006 to May 2008.

4.2.2. Experimental design

Four lucerne cultivars were planted in each of the three environments as a randomised complete block design with four replications. Cultivars (Plate 4.1) were DuPuits (winter-dormant), Grasslands Kaituna (semi winter-dormant), SARDI 7 (winter-active) and SARDI 10 (highly winter-active). Grasslands Kaituna, SARDI 7 and SARDI 10 represent recently released readily available cultivars, while DuPuits represents one of the check cultivars used in experimental line testing in Tasmania during the 1980s and 90s. Plots at Elliott were 3.5 × 7 m in size, while at Cambridge plots were 4.5 × 7 m. In all environments, to minimise edge effects a 0.5 m wide buffer of lucerne surrounded each plot.

Plate 4.1. Plots of lucerne just prior to harvest in September 2007 at Cambridge, Tasmania. Cultivars marked on the plate are DuPuits (DUP), Grasslands Kaituna (GK), SARDI 7 (S7) and SARDI 10 (S10).

4.2.3. Cultural practices

At each environment a seedbed was prepared over the winter of 2006. For primary cultivations, offset discs were used at Cambridge and a deep ripper was used at Elliott. A rotary hoe was used for secondary cultivations at both locations. During seedbed preparation 4 kg/ha of N (as urea, 46% N), 6 kg/ha of P (as molybdenum fortified single superphosphate) and 7 kg/ha of K (as muriate of potash) was applied based on the soil test results presented in table 4.1. This ensured that soil fertility was not limiting during crop establishment. To raise soil pH to a level conducive to lucerne production, 5.0 t/ha of lime was applied at Elliott and 1 t/ha of lime was applied at Cambridge after the primary cultivations.

Seed was drill sown at a rate of 15 kg pure live seed/ha at a depth of 15 mm and a row spacing of 150 mm. Planting at the Cambridge environment occurred on 31 October 2006 while planting at Elliott occurred on 17 January 2007. Seed planted at Cambridge was freshly inoculated with *Rhizobia* (Group AL; Becker Underwood

Pty Ltd, Somersby, NSW Australia) and lime coated after weighing. Bare seed was sown at Elliott, and then, at the emergence of the first true leaf, plots were spray inoculated in the evening with *Rhizobia*, followed by 20 mm irrigation to ensure that the inoculant was incorporated into the soil. At both Cambridge and Elliott, plots were irrigated from planting to the first defoliation, after which the irrigations were only maintained in the irrigated plots at Elliott. Irrigations occurred when soil water content was 20 mm below the DUL estimated using estimated ET and rainfall as described by Allen *et al.* (1998). An application of 20 mm was applied at each irrigation event. From planting to the first harvest 160 and 120 mm of water was applied at Cambridge and Elliott respectively. During the 2007/08 irrigation season 320 mm of water was applied to the irrigation environment at Elliott.

Plots at both locations were regularly scouted for weeds, pests and diseases. Weed pressure was alleviated using pre- and post-emergent proprietary herbicides. Trifluralin 480 (trifluralin 480 g/L) was applied during seedbed preparation at a rate of 1.7 L/ha. To remove weeds after planting, 2,4-DB 500 g/L; Buttress) and bromoxynil 200 g/L; Bromicide 200) were applied at a rate of 3 L/ha and 1.5 L/ha respectively, on 9 January 2007 at Cambridge, while Velpar DF was applied at a rate of 3 L/ha on 7 September 2007 at Elliott. Pest pressure from lucerne flea was alleviated at Elliott through 2 applications of omethoate 290 g/kg; Lemat) at a rate of 100 ml/ha on 29 November 2007 and 10 December 2007. No diseases were observed in any plot during the experiment.

Fertilisers were applied in the winter of 2007, based on soil tests taken in the autumn of 2007 and previous forage removal. At Elliott, 385 kg/ha of K in the form of potassium sulphate (42% K, 18% sulphur; S) and 160 kg/ha of P in the form of triple super phosphate (21% P) were applied. At Cambridge, 170 kg/ha of K in the form of potassium sulphate and 4 kg/ha of P in the form of single superphosphate were applied.

4.2.4. Measurements

Plant counts were undertaken at six weeks after planting. Plants were counted along three 1 m lengths of row within each plot. Plants per m^2 were then calculated using row spacing.

Harvests were timed to coincide with the elongation of crown buds. Within each environment crown bud elongation for all cultivars occurred within one week of each other, as such, all plots were harvested on the same day when elongation was observed in all plots. At each harvest, for each plot three 0.25 m^2 quadrats were defoliated to a height of 50 mm with hand shears, and the cut material gathered. The number of lucerne shoots were counted, and the sample was separated into three sub-samples of approximately equal size. The first sub-sample was weighed and dried to determine moisture content. Lucerne shoots in the second sub-sample were grouped based on morphological development stage (Kalu and Fick 1981). The final sub-sample was hand separated into lucerne leaves and stems. All samples were dried at 60^oC for 48 h and weighed to 0.01 g. Mass per shoot, number of shoots per m^2, MSW (Kalu and Fick 1981) as an assessment of maturity and leaf to stem ratio were calculated.

After the quadrate samples were taken, a central 6 × 1 m strip in each plot was cut to 50 mm with a reciprocating mower and the cut material was gathered and weighed to 0.01 kg. Dry matter yield was then calculated using moisture content. The plant frequency of lucerne plants within the central strip was determined using the 100 square grid method with 10 cm^2 quadrats as described by Lodge and Gleeson (1984). The total number of shoots and the number of crown shoots per plant was determined on an average of four plants that were destructively harvested from outside the central 6 × 1 m defoliated strip. The remainder of the foliage on the plot was then cut with a reciprocating mower to a height of 50 mm and discarded.

Volumetric SWC was monitored in the dryland and irrigated environments at Elliott using the gopher soil moisture profiling system (Dataflow Systems; Christchurch, New Zealand). Polyvinyl chloride access tubes were installed on 14 June 2007. Measurements in all plots were taken in 10 cm depth increments, to 110 cm, directly before and 24 hours after irrigation applications and at each defoliation. A measurement was taken at least every seven days.

Deep drainage (DD) for each regrowth period was estimated by summing the difference between SWC and DUL each time measured SWC was greater than the DUL during the regrowth period (Equation 4.1). Total estimated ET during each regrowth period was calculated using the difference between SWC at the start of the

regrowth period and at the end of the regrowth period, water applied through irrigation and rainfall (w), and the estimated water lost through deep drainage (Equation 4.2).

$$DD = \sum_{x=1}^{N} (SWC_x - DUL)$$

Equation 4.1: $\text{Where : IF} = SWC_x < DUL, \text{ Then } SWC_x = DUL$

x = a SWC measurement

N = total number of SWC made during the regrowth period.

Equation 4.2: $ET = SWC_1 - SWC_N + w - DD$

4.2.5. Statistical analysis

Data gathered from each environment were subjected to an ANOVA for each measured variable. Fishers LSD was used to identify the differences between cultivars where ANOVA identified a significant effect ($P < 0.05$). To determine the relative contribution of each yield component to yield, stepwise linear regressions were performed using the natural logarithm of yield as the response variable and the natural logarithm of mass per shoot and number of shoots per m^2 as potential predictor parameters. A log transformation was used as the shoots per m^2 variable contained counts covering a large range of values. An F value greater than 4 was the test criterion for the addition or removal of a parameter to or from the model. Stepwise linear regressions were performed at the main effect levels of environment and cultivar, as well as at the interaction level of environment by cultivar. Unless otherwise stated significance was accepted when $P < 0.05$. All statistical analyses were undertaken using Genstat 11th edition (VSN International Ltd, Hemel Hempstead, UK).

4.3. Results

4.3.1. Plant establishment

There were no significant differences among cultivar on the number of established plants in any of the environments (Table 4.2). Plant density at six weeks after planting averaged 24, 42 and 56 plants per m^2 at the Cambridge and Elliott dryland and irrigated environments respectively.

Table 4.2. Number of plants established (plants/m^2) of four lucerne cultivars (DuPuits, Grasslands Kaituna, SARDI 7 and SARDI 10) grown in three environments (dryland at Cambridge, dryland at Elliott and irrigated at Elliott) in Tasmania from February 2007 to June 2008.

	Dryland at Cambridge	Dryland at Elliott	Irrigated at Elliott
Cultivar		plants/m^2	
DuPuits	23	45	60
Grasslands Kaituna	29	35	50
SARDI 7	20	46	57
SARDI 10	23	43	57
LSD ($P < 0.05$)	ns†	ns	ns

ns†, no significant effect

4.3.2. Dry matter yield

Cultivar had a significant effect on total DM yield in both dryland environments. Grasslands Kaituna produced more DM than DuPuits over the length of the experiment in both dryland environments (Table 4.3). In the dryland Elliott environment, Grasslands Kaituna also produced more DM than SARDI 10. SARDI 10 produced more DM than DuPuits in the dryland environment at Cambridge. There was no difference ($P > 0.05$) in the yield of cultivars grown under irrigation at Elliott.

Table 4.3. Total dry matter yield (kg DM/ha) of four lucerne cultivars (DuPuits, Grasslands Kaituna, SARDI 7 and SARDI 10) grown in three environments (dryland at Cambridge, dryland at Elliott and irrigated at Elliott) in Tasmania from February 2007 to June 2008.

	Dryland at Cambridge	Dryland at Elliott	Irrigated at Elliott
Cultivar	kg DM/ha		
DuPuits	4227	12012	19776
Grasslands Kaituna	6054	13256	19738
SARDI 7	4969	12863	19471
SARDI 10	5750	11449	20498
LSD ($P < 0.05$)	1252	903	ns[†]

ns[†], no significant effect

Cultivar yield differences at individual harvests were not consistent within or between environments (Figure 4.2). At Cambridge, SARDI 10 produced more DM than DuPuits in December 2007 and January, March and June 2008 ($P < 0.05$). In June 2008, SARDI 10 also had a significantly greater ($P < 0.05$) DM yield than Grasslands Kaituna and SARDI 7. Grasslands Kaituna produced the most DM at Cambridge in February 2007.

In November 2007, under dryland conditions at Elliott, SARDI 10 produced significantly ($P < 0.05$) less DM than the other cultivars. At the June 2008 harvest, SARDI 7 and SARDI 10 had a greater DM yield compared to DuPuits.

With irrigation at Elliott, SARDI 10 produced more DM than DuPuits or Grasslands Kaituna at the May and September 2007 harvests. This pattern was reversed for the November 2007 harvest where DuPuits produced more DM than SARDI 7 and SARDI 10, and Grasslands Kaituna produced more DM than SARDI 7.

4.3.3. Yield components

Cultivar had a significant effect on mass per shoot at both Elliott environments, while, at Cambridge, cultivar had a significant effect on number of shoots per plant and the proportion of crown shoots making up the sward. Under dryland conditions at Elliott, Grasslands Kaituna and SARDI 7 had a greater mass per shoot compared to DuPuits (Table 4.4). Grasslands Kaituna also had a greater mass per shoot

compared to SARDI 10. At Elliott, under irrigated conditions, SARDI 7 had the greatest mass per shoot.

In the Cambridge environment, SARDI 10 had a greater number of shoots per plant than DuPuits and Grasslands Kaituna, and Grasslands Kaituna had the highest proportion of crown shoots in the sward. Crown shoots were always the most numerous shoot type, comprising more than 50% of the total number of shoots in all of the environments. Both the number of shoots per m^2 and plant frequency were unaffected by cultivar in any environment. Averaged over the cultivars and harvests, the number of shoots per m^2 was 550, 540 and 549, and plant frequency was 57, 67 and 64% at Cambridge, Dryland at Elliott and Irrigated at Elliott, respectively. The effect of cultivar on each yield component at each harvest is presented in Figure 4.3.

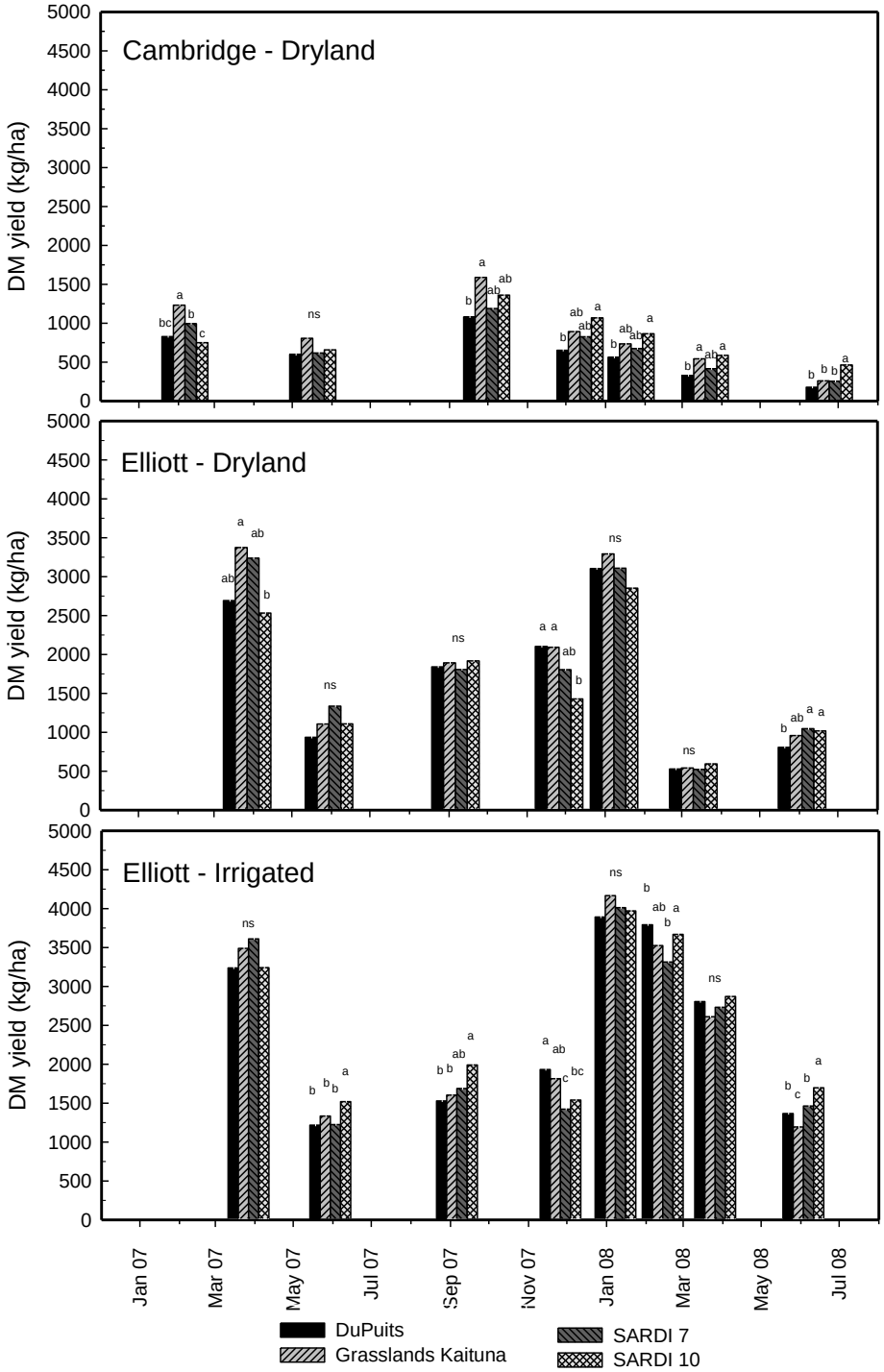

Figure 4.2. Dry mater yield (kg DM/ha) at each harvest of four lucerne cultivars (DuPuits, Grasslands Kaituna, SARDI 7 and SARDI 10) grown in three environments; dryland at Cambridge, dryland at Elliott and irrigated at Elliott, in Tasmania, from February 2007 to June 2008. Bars with different letters within the same harvest are significantly different using LSD (P = 0.05) and ns signifies no significant effect.

Table 4.4. Mass per shoot (g), number of shoots per plant and proportion of crown shoots in the sward (%) averaged over all harvests for four lucerne cultivars (DuPuits, Grasslands Kaituna, SARDI 7 and SARDI 10) grown in three environments (dryland at Cambridge, dryland at Elliott and irrigated at Elliott) in Tasmania, from February 2007 to June 2008.

	Dryland at Cambridge	Dryland at Elliott	Irrigated at Elliott
Cultivar		Mass per shoot (g/shoot)	
DuPuits	0.28	0.42	0.60
Grasslands Kaituna	0.30	0.52	0.66
SARDI 7	0.27	0.50	0.74
SARDI 10	0.35	0.44	0.61
LSD ($P < 0.05$)	ns[†]	0.07	0.09
Cultivar		Shoots per plant	
DuPuits	3.7	3.0	4.1
Grasslands Kaituna	3.6	3.3	4.4
SARDI 7	4.6	3.1	4.1
SARDI 10	5.6	2.6	3.8
LSD ($P < 0.05$)	1.4	ns	ns
Cultivar		Percentage of crown shoots in the sward	
DuPuits	76%	84%	87%
Grasslands Kaituna	85%	85%	82%
SARDI 7	76%	86%	86%
SARDI 10	83%	90%	89%
LSD ($P < 0.05$)	7%	ns	ns

ns[†], no significant effect

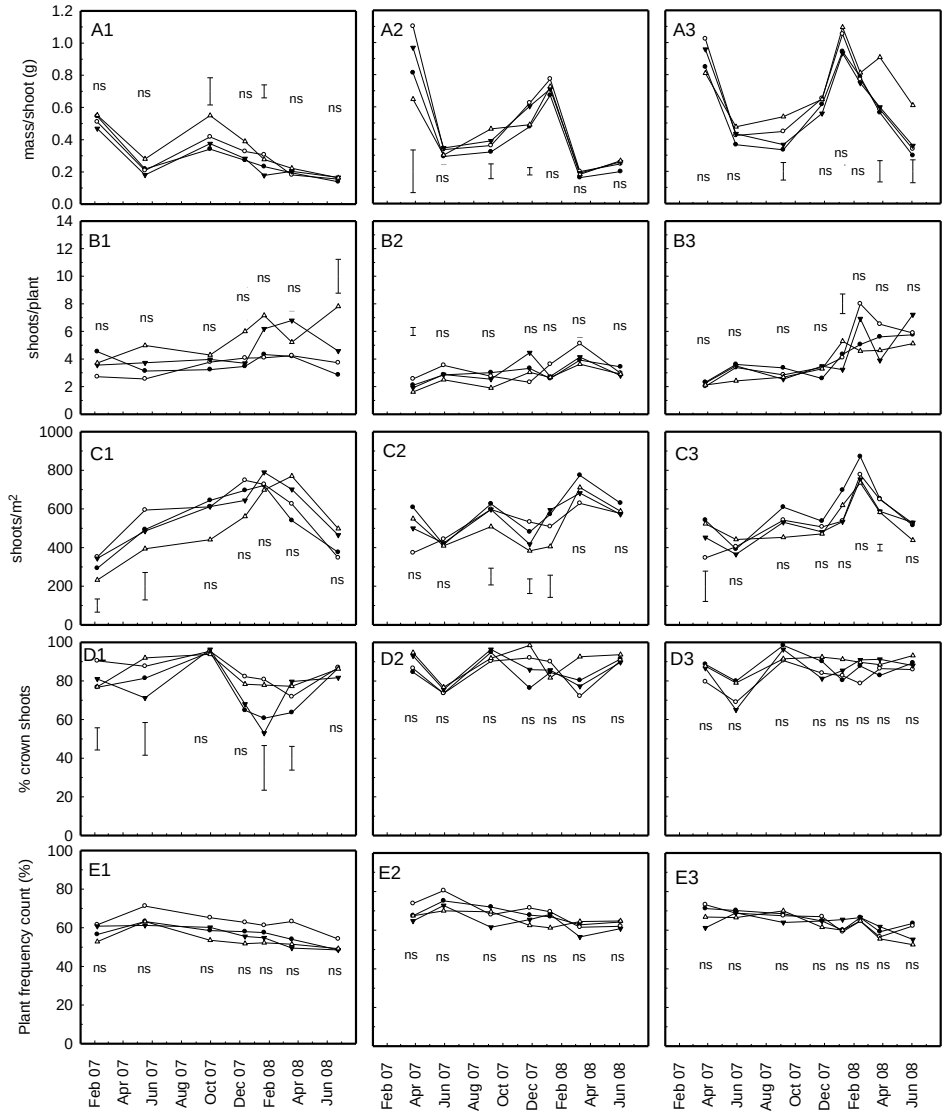

Figure 4.3. Mass per shoot (g), number of shoots per plant, number of shoots per m^2, proportion of crown shoots in the sward (%) and plant frequency (%) at each harvest for four lucerne cultivars (DuPuits: ○; Grasslands Kaituna: ●; SARDI 7: ▼; SARDI 10: △) grown in three environments (dryland at Cambridge, panels A1, B1, C1, D1, and E1; dryland at Elliott, panels A2, B2, C2, D2 and E2; and irrigated at Elliott, panels A3, B3, C3, D3 and E3) in Tasmania, from February 2007 to June 2008. Error bars for each harvest represent the LSD at $P = 0.05$ and ns represents no significant effect.

4.3.4. Relative influence of mass per shoot and number of shoots per m^2 on dry matter yield

Mass per shoot consistently had a greater influence on the linear model explaining DM yield than did number of shoots per m^2 (Table 4.5). In the dryland environment at Elliott, the number of shoots per m^2 failed to significantly improve the model explaining yield, with mass per shoot adequately explaining variability at this level.

At the interaction level of environment by cultivar, the number of shoots per m^2 did not make an improvement to the model explaining DM yield for any cultivar in the dryland environment at Elliott or for SARDI 7 at Cambridge. Where number of shoots per m^2 improved the model for Cambridge or under irrigation at Elliott, its influence in the model was always less than that of mass per shoot.

4.3.5. Mean stage weight and leaf to stem ratio

Mean stage weight was significantly affected ($P < 0.05$) by cultivar under dryland and irrigated conditions at Elliott. In both these environments DuPuits had the lowest MSW. Under irrigation at Elliott, Grasslands Kaituna and SARDI 7 had a lower MSW compared to SARDI 10 (Table 4.6). The range of MSW observed at Cambridge was greater than at either Elliott environments. However, no significant difference between cultivars was observed at Cambridge highlighting a greater viability for this characteristic in that environment.

Cultivar significantly affected leaf to stem ratio in all three environments ($P < 0.05$). In the Cambridge and dryland Elliott environments, DuPuits had the highest leaf to stem ratio, while under irrigation at Elliott both DuPuits and Grasslands Kaituna had the highest leaf to stem ratio.

Table 4.5. Regression coefficients from stepwise linear regressions of the natural log (ln) of yield components and yield at the main effect levels of environment and cultivar, and the interaction level of environment by cultivar. Values in parenthesis are the standard errors associated with each coefficient.

Environment by cultivar	Regression coefficients		R^2
	ln shoot mass	ln shoots per m^2	
Dryland Cambridge	0.93 (0.07) ***	0.64 (0.10) ***	0.632
Dryland Elliott	0.98 (0.05) ***	ns	0.802
Irrigated Elliott	0.75 (0.05) ***	0.53 (0.08) ***	0.713
DuPuits	1.15 (0.07) ***	0.77 (0.15) ***	0.775
Grasslands Kaituna	1.11 (0.06) ***	0.54 (0.13) ***	0.816
SARDI 7	1.08 (0.06) ***	0.43 (0.15) **	0.786
SARDI 10	1.04 (0.06) ***	0.42 (0.10) ***	0.785
Dryland Cambridge by DuPuits	0.88 (0.15) ***	0.79 (0.21) ***	0.614
Dryland Cambridge by Grasslands Kaituna	0.89 (0.14) ***	0.68 (0.21) **	0.637
Dryland Cambridge by SARDI 7	0.87 (0.17) ***	ns†	0.497
Dryland Cambridge by SARDI 10	0.81 (0.10) ***	0.46 (0.11) ***	0.722
Dryland Elliott by DuPuits	1.00 (0.12) ***	ns	0.740
Dryland Elliott by Grasslands Kaituna	0.98 (0.09) ***	ns	0.828
Dryland Elliott by SARDI 7	0.98 (0.08) ***	ns	0.829
Dryland Elliott by SARDI 10	0.98 (0.10) ***	ns	0.794
Irrigated Elliott by DuPuits	0.75 (0.08) ***	0.65 (0.14) ***	0.842
Irrigated Elliott by Grasslands Kaituna	0.93 (0.10) ***	0.35 (0.17) *	0.731
Irrigated Elliott by SARDI 7	0.68 (0.10) ***	0.56 (0.18) **	0.655
Irrigated Elliott by SARDI 10	0.67(0.15) ***	0.61 (0.20) **	0.605

* significant contribution of the yield component to the model at $P < 0.05$
** significant contribution of the yield component to the model at $P < 0.01$
*** significant contribution of the yield component to the model at $P < 0.001$
ns† no significant ($P > 0.05$) contribution from the yield component to the model.

Table 4.6. Mean stage weight (MSW) and leaf to stem ratio averaged over all harvests for four lucerne cultivars (DuPuits, Grasslands Kaituna, SARDI 7 and SARDI 10) grown in three environments (dryland at Cambridge, dryland at Elliott and irrigated at Elliott) in Tasmania from February 2007 to June 2008.

	Dryland at Cambridge	Dryland at Elliott	Irrigated at Elliott
Cultivar		MSW	
DuPuits	2.0	2.0	2.1
Grasslands Kaituna	2.6	2.4	2.4
SARDI 7	2.2	2.3	2.3
SARDI 10	2.9	2.5	2.6
LSD ($P < 0.05$)	ns[†]	0.3	0.1
Cultivar		Leaf to stem ratio	
DuPuits	1.93	1.57	1.14
Grasslands Kaituna	1.55	1.36	1.14
SARDI 7	1.69	1.28	0.99
SARDI 10	1.28	1.15	0.85
LSD ($P < 0.05$)	0.27	0.17	0.14

ns[†] no significant effect

4.3.6. Estimated evapo-transpiration

There was no significant effect from cultivar on estimated ET under either the irrigated or dryland environments at Elliott. As expected, estimated ET was greatest in the irrigated environment compared to the dryland environment (Table 4.7), with the greatest difference observed during summer.

Table 4.7. Total estimated evapotranspiration (ET) and average daily ET for each regrowth period of lucerne grown at Elliott under dryland or irrigated conditions. Data is the average of four cultivars.

	Days of regrowth	Total ET (mm)	Average daily ET (mm/day)
Harvest date		Dryland	
6/09/2007	98	223	2.27
26/11/2007	81	291	3.60
8/01/2008	43	146	3.39
10/03/2008	62	93	1.49
3/06/2008	85	169	1.98
Total	369	921	2.50
Harvest date		Irrigated	
6/09/2007	98	225	2.30
26/11/2007	81	299	3.69
8/01/2008	43	192	4.46
14/02/2008	37	144	3.89
26/03/2008	41	143	3.50
3/06/2008	69	225	3.27
Total	369	1228	3.33

4.4. Discussion

While a genotype by environmental interaction on yield was observed in this study there was no interaction on the relative contribution of each yield component to yield or plant morphology. Cultivar yield differences only occurred in the environments that were subjected to water deficits, suggesting that the genetic differences for yield between these four cultivars are only expressed when plants are under water stress, with Grasslands Kaituna having the greatest yield in both dryland environments. While previous assessments from North America in arid environments have identified genotype by environmental interaction in response to water deficit, these responses have been inconsistent between environments (Orloff and Hanson 2008). In Chapter 3 it was identified that yield differences between winter dormancy types are only exhibited in low yield potential environments. The results from the current field experiment confirm that the genotype by environmental interaction for yield in cool temperate environments are repeatable and hence have the potential to be exploited (Cooper *et al.* 2006; Annicchiarico 2009). In this study the most winter dormant cultivar DuPuits had a similar yield to SARDI 10 in both dryland environments. DuPuits is an older and relatively unimproved cultivar, whereas the other cultivars are all recent commercial releases. Improvements in the genetic potential for stress tolerance of the other cultivars compared to DuPuits may account for this contradiction between the current study and the study presented in Chapter 3.

Differences in root system structure (Bennett and Doss 1960; McIntosh and Miller 1981; Carter *et al.* 1982; Salter *et al.* 1984) and soil water uptake pattern (Hattendorf *et al.* 1990) suggests that, under water limiting conditions, winter-dormant cultivars are able to access more soil water than winter-active cultivars. However, there was no cultivar difference in the estimated ET of any of the cultivars when grown in the dryland or irrigated environments at Elliott. As such the better performance of Grasslands Kaituna compared to SARDI 10 at Elliott under dryland conditions was not due to differences in plant water uptake, but differences between these two cultivars in their physiological adaptation to moisture deficit stress.

Genotype has been identified to influence yield in environments prone to stand losses (Lamb *et al.* 2006). Lamb *et al.* (2006) suggested that stand loss from disease incidence caused differences in yield between cultivars. However, no stand

decline was observed in any environment (Figure 4.3, panels E1, E2 and E3), although yield potential was reduced in the dryland environments due to summer water deficits. In Chapter 3 pest and disease incidence was found to have minimal impact on genotypic differences in yield. The results of the experiments described in the current and previous chapter is evidence that stand decline and the expression of yield differences between cultivars were not causally related, and that both were a response to less than ideal conditions for production.

The effect of cultivar on mass per shoot in the current experiment is inconsistent with previous reports. For example, studies by Volenec *et al.* (1987) under field conditions and by Leach (1969) in pots, found that highly winter-active cultivars had a greater mass per shoot compared to more winter-dormant cultivars. In this experiment, under dryland conditions at Elliott, Grasslands Kaituna (a semi winter-dormant cultivar) had the greatest mass per shoot, while under irrigated conditions at Elliott, SARDI 7 (a winter-active cultivar) had the greatest mass per shoot. The cultivars used in previous studies (Leach 1969; Volenec *et al.* 1987) are not the same as the cultivars used in the current study, and hence the considerable genetic differences in plant morphology between lucerne cultivars, as highlighted by Julier *et al.* (2000), is a likely cause for this discrepancy.

Of the yield components monitored, the seasonal pattern of mass per shoot was similar to that of yield. The number of shoots per plant remained stable or increased, even as forage yields decreased in autumn. The number of shoots per m^2 increased under all environments in early autumn, while yield and mass per shoot decreased, suggesting that mass per shoot is a better predictor of yield. This was confirmed by stepwise regression analysis (Table 4.5), where mass per shoot had the greatest positive influence on yield at both the environment and cultivar levels, as well as the interaction level between the two. Path analysis revealed that increases in mass per shoot in young lucerne stands were associated with increased yield with improved soil fertility (Berg *et al.* 2005), and it was not until the 6^{th} year after planting that the number of shoots per m^2 were positively associated with yield (Berg *et al.* 2007). By controlling plant population using transplanting instead of direct seeding, Volenec *et al.* (1987) concluded that greater numbers of shoots per plant do not lead to higher yields.

Stepwise regressions in the current study showed that increasing shoot densities has a positive impact on yield in some situations. However, this influence was always less than that of mass per shoot. In the dryland Elliott environment, mass per shoot was the only yield component with a significant influence on yield. Summer drought in the dryland Cambridge environment may have limited mass per shoot to the extent that the number of shoots per area exerted a greater influence on yield than would normally be the case.

The maintenance of a large proportion of crown shoots throughout the experiment was a result of the cutting management. Elongation of crown buds was used to time defoliation - a best management practice for lucerne crops in Australia (Gramshaw *et al.* 1981; Lodge 1991; Gramshaw *et al.* 1993; Lowe *et al.* 2002). Crown shoots are higher yielding than axial shoots and hence are the more desirable shoot type (Leach 1968; 1971; Gramshaw *et al.* 1981). Harvesting at crown bud elongation ensured that a high number of crown shoots was maintained.

Where differences in leaf to stem ratio between the cultivars occurred, winter-active cultivars had lower ratios in all environments. A similar relationship between winter activity and leaf to stem ratio has been reported for the Mediterranean (Humphries and Hughes 2006) and subtropical (Lodge 1986) environments of Australia. Differences in stem thickness and the leaf to stem ratio between lucerne cultivars result in differences in forage digestibility (Volenec *et al.* 1987). The absence of a genotype by environmental interaction on the forage quality parameters means that winter-dormant cultivars would be expected to have herbage of higher quality across a broad range of environments.

The current study has identified that while some genotype by environment interactions are present for lucerne yield components, mass per shoot is consistently the yield component that has greatest influence on yield in establishing lucerne crops, and that genetic differences for yield amongst the cultivars evaluated were only exhibited under conditions of limited water. Identification of factors affecting DM accumulation of shoots under water limited conditions and how genotype influences these factors are required to further understand and maximise the advantage of exploiting this genotype by environmental interaction.

CHAPTER 5

Effect of summer irrigation on seasonal changes in taproot reserves and the expression of winter dormancy/activity in four contrasting lucerne cultivars.

5.1. Introduction

The growth and production of lucerne across a range of cool temperate environments is affected by genotype (Chapters 3 and 4) with winter dormant genotypes adapted to low yield potential environments and winter active cultivars adapted to high yield potential environments. Irrigation is used to enhance lucerne growth compared to what would be produced when plants are grown under dryland conditions (Brown and Tanner 1983; Collino *et al.* 2005; Chapter 4) and as such is a key factor determining the yield potential of an environment. Despite the considerable influence that irrigation has on lucerne growth, there is little information regarding the effect of irrigation on seasonal changes in taproot carbohydrate and N reserves. Furthermore the limited information available gives no indication as to whether this effect would be influenced by winter activity as conditioned by plant genotype.

Differences between winter activity genotypes in the levels of taproot carbohydrate and N reserves have been observed during autumn and winter in North American environments (Cunningham and Volenec 1998; Cunningham *et al.* 1998) and during late summer in the Queensland subtropics (Gramshaw *et al.* 1993). In addition to the influence of genotype, taproot carbohydrate and N reserves throughout the growing season are influenced by climatic conditions (Li *et al.* 1996; Teixeira *et al.* 2007c), defoliation practices (Avice *et al.* 1996b; Haagenson *et al.* 2003a; Teixeira *et al.* 2007c) and soil fertility (Li *et al.* 1997; Qamar *et al.* 2006; Berg *et al.* 2009).

As irrigation is expected to alter the pattern of plant assimilate partitioning, the genetic and phenotypic expression of cold acclimation and winter dormancy may also be affected. This would have implications for the management of lucerne stands during autumn and winter to minimise abiotic stress over winter, and ensure rapid growth in spring. This could potentially explain the genotype by environmental interaction between irrigated and dryland environments identified in Chapter 4. This chapter will test the hypothesis that the genotype influence on taproot carbohydrate and N reserves and the abundance of key ribonucleic acid (RNA) transcripts associated with cold acclimation will be altered by summer irrigation. The objective of this chapter is to evaluate seasonal changes in taproot reserves and the phenotypic and genetic expression of winter dormancy under both irrigated and dryland conditions for four lucerne cultivars with contrasting levels of winter activity.

5.2. Materials and Methods

5.2.1. Sample collection and measurements

The irrigated and dryland environments planted with DuPuits, Grasslands Kaituna, SARDI 7 and SARDI 10 lucerne at Elliott (Plate 5.1), and described in Chapter 4, were used as a source of taproot tissue. At each defoliation from 26 November 2007 onwards, taproots separated from the crowns of plants destructively harvested (Chapter 4). Plants were excavated to a depth of 200mm using a 200mm diameter corer and were washed free of soil in cold water. Taproots were separated from the crown at the lowest crown bud or crown branch and frozen on dry ice for biochemical analysis. To capture biochemical changes in taproots that are associated with cold acclimation, an additional taproot sampling occurred on 29 April 2008 without defoliation. At this sampling and the sampling that occurred with defoliation on 3 June 2008, additional plants were destructively sampled from a second core. After washing these additional samples, the top 50 mm of the taproot was diced and frozen in liquid nitrogen for RNA extraction. All taproot tissue was stored at -80^{o}C. Tissue for biochemical analysis was freeze dried and ground through a 1 mm screen. Tissue for RNA isolation was ground to a fine powder in liquid nitrogen with a

mortar and pestle. At the 29 April and 3 June 2008 samplings, sward height was measured five times along the centre row of each plot and the measurements averaged.

Plate 5.1. Irrigated (left) and dryland (right) lucerne plots during February 2008 at Elliott, Tasmania.

5.2.2. Tissue biochemical and molecular analysis

Soluble sugars were extracted from 30 mg of freeze dried root tissue using 80% ethanol, as described by Li *et al.* (1996), and the concentration of soluble sugars in the extract determined with anthrone (Van Handel 1968) using glucose as a standard. The ethanol-extracted residue was dried at 55^{o}C for 24 hrs. Starch in the sugar-extracted residue was digested to glucose in a 100 mmol/L mono basic sodium phosphate (NaH_2PO_4) buffer (pH 5.3) containing 40 units of α-amylase (Sigma Aldrich catalogue No.: 4551, St. Louis, MO, USA) and 0.2 units of amyloglucosidase (Sigma Aldrich catalogue No.: 1602). The amount of glucose present after digestion was determined using glucose oxidase (Trinder assay; Diagnostic Chemicals Ltd. Charlottetown, PE, Canada) as described by Li *et al.* (1996). Starch concentration was estimated as 0.9 × glucose concentration. Total non-structural carbohydrates were considered to be the sum of soluble sugars and starch.

An extraction buffer with a pH of 6.8 and containing 100 mmol/L NaH_2PO_4 (pH 6.8), 1 mmol/L phenylmethylsulfonyl flouride and 10 mmol/L 2-mercaptoethanol was used to extract soluble proteins and amino acids from 30 mg of lyophilised root tissue, following the procedure described in Cunningham *et al.* (1998). Samples were kept at 4^{o}C throughout the entire extraction. Protein concentration in the extract was determined using the Bradford protein dye binding method (Bradford 1976; Cunningham *et al.* 1998). Amino-acid concentration in the extract was determined using ninhydrin with glycine as a standard (Rosen 1957). All taproot reserve concentrations are expressed on a dry weight basis.

For sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, protein extract was mixed one-to-one with SDS-PAGE sample loading buffer (0.125 mol/L Tris pH 6.8, 22.5% v/v glycerol, 0.004% v/v bromophenol blue, 1.42 mol/L 2-mecaptaethanol, 0.114 mol/L sodium dodecylsulphate). Twenty micro grams of protein from three replications were separated in 1.5 mm thick gels containing 15% (w/v) acrylamide (Laemmli 1970) and stained with Coomassie Brilliant Blue R-250. Gels were scanned using white light on a flatbed scanner with a transparent materials adaptor (Hewlett Packard Scanjet 4890, Hewlett Packard, Palo Alto, CA, USA). The transparent materials adaptor performs the same function as the modification of a flat bed scanner described by Tan *et al.* (2007) in that it allows for the analysis of light transmitted through the gel. Optical intensity of bands corresponding to the low, middle and high molecular weight lucerne VSPs were determined using image analysis software (IMAGE J, National Institute of Health, Bethesda, MD, USA).

To confirm that the analysed bands did correspond to the VSPs, selected samples of extracted protein were subjected to immuno-detection, using western blotting. Extracted protein (2.5 µg) from selected samples was separated on SDS-PAGE gels as described above. Proteins were transferred on to nitrocellulose membranes (Protoban BA83, Schleicher& Scultell, Keene, NH, USA) as described by Towbin *et al*. (1979). All subsequent probing with antibodies and washing occurred with gentle agitation at room temperature. Non-specific binding sites were blocked by immersing membranes for 30 minutes in tris-buffed saline (TBS; 10

mmol/L Tris, 150 mmol/L sodium chloride; NaCl, pH 8.0) containing 0.15% (v/v) Tween 20 (TBST). Membranes were incubated for 90 minutes with antibodies raised to the low or middle molecular weight lucerne VSPs (Cunningham and Volenec 1996). Antibodies to the VSPs were diluted at 1:10 000 in TBST. After primary antibody incubation, membranes were washed five times in TBST for a total of 25 minutes, and then immersed for 90 minutes in TBST containing a 1:3000 dilution of goat- anti-rabbit antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were then washed seven times for a total of 35 minutes in TBST, three times for a total of 15 minutes in TBS, and once for five minutes in alkaline phosphatase buffer (100 mmol/L Tris; pH 9.5, 100 mmol/L NaCl and 5 mmol/L Magnesium Chloride; $MgCl_2$). A solution of 0.7 mmol/L 5-bromo-4-chromo-3-indolyl phosphate ρ-Toluidine salt and 0.2 mmol/L nitro blue tetrazolium in alkaline phosphatase buffer was used to visually detect the secondary antibodies (Blake *et al.* 1984).

Total ribonucleic acid (RNA) was extracted using phenol and chloroform as described by Gana *et al.* (1998). Extracted RNA concentration and quality (260/280 nm ratio) was spectrometrically determined with triplicate samples (Nanodrop 1000, Nanodrop Technologies Inc. Wilmington, DE, USA) and its integrity checked by separating 20 μg in 1.5% agarose-formaldehyde gels containing ethidium bromide. RNA was visualised with exposure to UV light (Gel Doc 2000, Bio-Rad Laboratories). Northern blots were created by the overnight transfer of RNA from gels onto nylon membranes (Zeta Probe Membranes, Bio-Rad Laboratories), UV imaged as described for the agarose gels, and UV cross-linked using procedures identical to those described by Gana *et al.* (1998). Northern blots were pre-hybridised under constant rotation at 42^{o}C for 4 hours in 20 mL of hybridisation buffer (50% v/v formamide, 0.12 mol/L dibasic sodium phosphate; Na_2HPO_4, pH 7.2, 0.25 M NaCl, 7% w/v sodium dodecylsulphate; SDS, and 1 mmol/L ethylenediaminetetraacetic acid; EDTA). Blots were probed using the complementary deoxyribonucleic acid (cDNAs) outlined in Table 5.1. Double stranded cDNA was randomly primed with deoxycytidine triphosphate labeled with radioactive phosphorus-32 ([^{32}P]dCTP; Feinberg and Vogelstein 1983) and

hybridised to Northern blots with constant rotation at 42^{o}C for 20 to 24 hours in hybridisation buffer. Blots were washed for 20 minutes in 0.3 mol/L NaCl, 30 mmol/L sodium citrate buffer (2X SSC) containing 0.1% w/v SDS with constant rotation at 42^{o}C. Two further washes contained 0.5X or 0.1X SSC with 0.1% (w/v) SDS. Following washing, blots were wrapped in plastic wrap and exposed to X-ray film (Fuji, Tokyo, Japan) at -80^{o}C. After exposure, probes were stripped from the blots by washing twice in a 0.1X SSC solution containing 0.5% w/v SDS at 95^{o}C under constant rotation. Blots were exposed to X-ray film to confirm the removal of probes before being re-probed. X-ray films were developed using a Konica SRX-101A Medical Film Processor (Konica Minolta, Wayne NJ, USA), scanned using a flat bed scanner (Hewlett Packard Scanjet 4890, Hewlett Packard) and the optical intensity of lanes on both films and blot images determined using image analysis software (IMAGE J, National Institute of Health). Optical intensity data from films was normalized using optical intensity data from their respective Northern blots.

Table 5.1. Probe ID, gene names, Genbank accession numbers, sequences length in base pairs (bp), and associated references of cDNA sequences used to probe Northern blots.

Probe ID	Gene name	Genbank accession number	Probe length (bp)	Comment/Reference
8c11a*	CAR1	AF072932	731 bp	(Haagenson *et al.* 2003a)
BC-2E 40a	Cas18	L07516	677 bp	Sequence homology to Cas18 (Wolfraim *et al.* 1993; Haagenson *et al.* 2003a)
BN1-12a3	Cas17/18	L13415 / L07516	610 bp	Sequence homology to both Cas17 and Cas18 (Wolfraim and Dhindsa 1993; Wolfraim *et al.* 1993; Haagenson *et al.* 2003a)
cyclo-bN1I1c	Sucrose synthase	O24301	1078 bp	Probe has 89% amino acid identity with the N terminus of pea (*Pisum sativum*) sucrose synthase (Berg *et al.* 2009)
RNT-26	High molecular weight VSP	AF530579	1322 bp	(Volenec *et al.* 2002; Berg *et al.* 2009)
pMSbA1	β-amylase	AF026217	1671 bp	(Gana *et al.* 1998; Berg *et al.* 2009)

5.2.3. Statistical analysis

Due to an absence of replication of irrigation treatment and differences in sampling dates between irrigated and dryland plots, data from each water treatment were analysed separately. Taproot biochemical data was subjected to an ANOVA of a split plot in time design with randomised complete blocks. Plant height and normalised optical density from Northern blot analysis was subjected to an ANOVA of a split plot in time design with randomised complete blocks. Where the ANOVA identified significant effects, differences between means were identified using Fishers LSD. Unless otherwise stated, significance was accepted when $P < 0.05$. All statistical analyses were completed using Genstat, 11th edition (VSN International Ltd, Hemel Hempstead, UK).

5.2.4. Climatic and soil water observations

Average monthly temperatures were lowest in June 2007 (a minimum of 2.0^{o}C; Figure 4.1), highest in February 2007 (a maximum of 23.1^{o}C; Figure 4.1) and similar to the long-term averages for the location (Figure 2.1). Rainfall exceeded estimated evapo-transpiration from May to October 2007, in December 2007, and from April to June 2008. Rainfall was below the long-term average for this location during November and December 2006, from February to April, and in June, July, October, and November of 2007, and January to April and June of 2008 (Figure 4.1).

Following the initiation of irrigation on 28 November 2007 volumetric SWC measured with the gopher soil moisture profiling system was greater under the irrigated conditions (Figure 5.1) when compared to dryland conditions. This continued throughout the summer and into autumn before SWC under both water regimes returned to the drained upper limit by 3 June 2008.

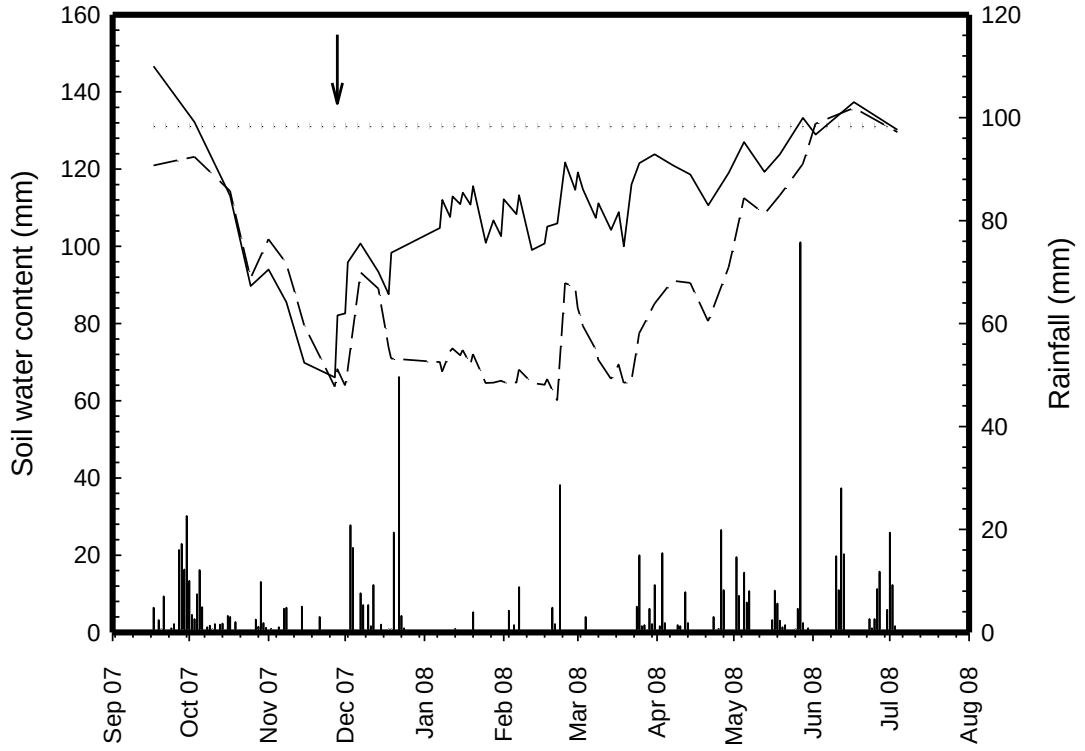

Figure 5.1. Volumetric soil water content (SWC; mm) to a depth of 400 mm under lucerne grown dryland (broken line) or irrigated conditions (solid line) and daily rainfall (bars) at Elliot Tasmania (lines represent the average of four cultivars) between 17 September 2007 and 7 August 2008. The dotted horizontal line represents the drained upper limit of the soil profile, and the arrow indicates when irrigation applications began for the 2007/08 season.

5.3. Results

5.3.1. Autumn Sward height

Sward height on 29 April and 3 June 2008 was affected by cultivar ($P < 0.001$) under both water regimes. SARDI 10 had the highest sward height under both water

regimes (Figure 5.2). Under irrigated conditions, Grasslands Kaituna had the lowest sward height, while, under dryland conditions, DuPuits had the lowest sward height.

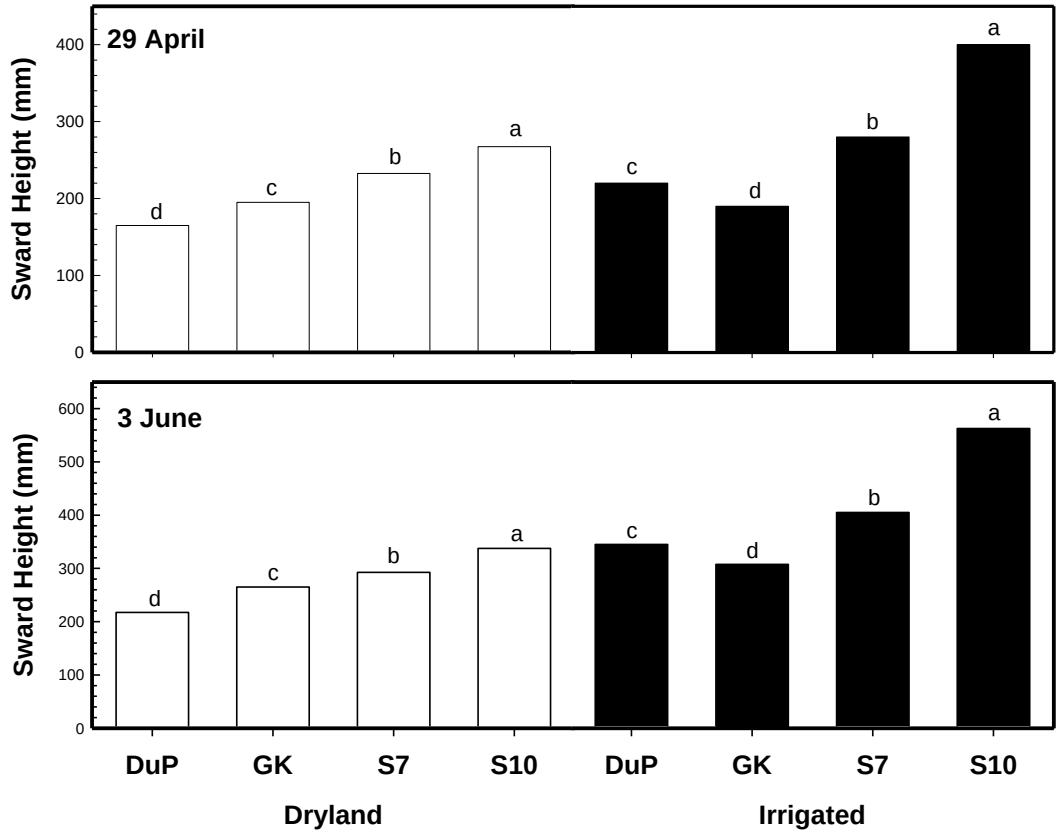

Figure 5.2. Sward height (cm) on 29 April 2008 and 3 June 2008 of DuPuits (DuP) Grassland Kaituna (GK), SARDI 7 (S7), and SARDI 10 (S10) lucerne grown under irrigated or dryland conditions at Elliott, Tasmania. Bars from cultivars with different letters within the same water regime and date are significantly different, using LSD at the 0.05 probability level.

5.3.2. Taproot biochemical composition

Cultivar affected ($P < 0.01$) TNC concentration under both irrigated and dryland conditions, while starch and sugar concentrations were only affected by cultivar under irrigated conditions. Under dryland conditions SARDI 10 had the greatest concentration of taproot TNC compared to the other cultivars (Table 5.2). Under irrigated conditions the lower taproot starch concentrations in SARDI 7 led to its lower TNC concentrations when compared to the other three cultivars. Taproot

soluble sugar concentrations were lowest in SARDI 10 plants grown under irrigation (Table 5.2).

Table 5.2. Cultivar differences (averaged across sampling dates) in total non-structural carbohydrate (TNC), soluble sugar, starch, and amino acid concentrations in taproots of lucerne grown under dryland (average of five samplings) or irrigated (average of six samplings) conditions at Elliott, Tasmania.

	Sugar concentration (mg/g)	Starch concentration (mg/g)	TNC concentration (mg/g)	Amino acid concentration (mmol/g)
Cultivar		Dryland		
DuPuits	95.9	318	414	0.279
Grasslands Kaituna	99.2	314	413	0.287
SARDI 7	90.1	327	417	0.299
SARDI 10	91.5	361	453	0.284
LSD (P = 0.05)	†ns	ns	24	ns
Cultivar		Irrigated		
DuPuits	92.7	288	381	0.245
Grasslands Kaituna	93.2	280	373	0.248
SARDI 7	94.0	252	346	0.252
SARDI 10	82.8	286	369	0.301
LSD (P = 0.05)	6.9	24	20	0.026

†ns: no significant effect

Seasonal changes of TNC, starch and soluble sugar concentrations in taproots were similar for all cultivars. Taproot TNC and starch concentrations declined throughout the season in plants grown under irrigation (Table 5.3). Under both water regimes taproot sugar concentrations increased throughout 2008, leading to maximum concentrations by 3 June in plants grown under irrigated conditions and by 29 April in those grown under dryland conditions (Table 5.3).

Table 5.3. Changes in soluble sugar, starch, total non-structural carbohydrate (TNC), and amino acid concentrations on a dry weight basis in taproots from 26 November 2007 to 3 June 2008 for lucerne grown under dryland or irrigated conditions at Elliott, Tasmania. Results are the average of four cultivars.

	Sugar concentration (mg/g)	Starch concentration (mg/g)	TNC concentration (mg/g)	Amino acid concentration (mmol/g)
Sampling date		Dryland		
26/11/2007	84.7	356	441	0.275
08/01/2008	86.5	336	426	0.314
10/03/2008	94.4	352	447	0.229
29/04/2008	105.9	289	399	0.290
03/06/2008	99.4	316	416	0.327
LSD (P = 0.05)	10.8	31	26	0.027
Sampling date		Irrigated		
26/11/2007	79.6	352	438	0.255
08/01/2008	66.0	342	408	0.283
14/02/2008	87.7	282	370	0.229
26/03/2008	97.0	254	351	0.281
29/04/2008	105.5	173	278	0.239
03/06/2008	108.2	257	365	0.281
LSD (P = 0.05)	12.5	43	39	0.038

There was no interaction between cultivar and sampling date under either water regime. Amino acid concentration was affected by cultivar ($P < 0.01$; Table 5.2) and sampling date (Table 5.3) under irrigated conditions, while, under dryland conditions, only sampling date affected ($P < 0.001$) amino acid concentration. Under irrigated conditions SARDI 10 had the greatest amino acid concentration in taproots compared to the other cultivars (Table 5.2). Concentration of amino acids (averaged over the four cultivars) decreased in late summer under both water regimes (Table 5.3).

Under dryland conditions there was a cultivar by sampling date interaction effect on soluble protein concentration where taproot soluble protein concentrations remained constant for all cultivars except for DuPuits which showed a significant

increase in taproot protein concentration between the March and May, as well as the May and June, sampling dates (Figure 5.3). Grasslands Kaituna taproots contained greater concentrations of soluble protein when compared to SARDI 10 at the 26 November sampling; the other three cultivars at the 8 January sampling date; and DuPuits at the 10 March sampling date (Figure 5.3).

Under irrigated conditions, sampling date was the only factor that had an effect on taproot soluble protein concentration (Figure 5.3). When soluble protein concentrations were averaged across the four cultivars, concentrations declined between 14 February and 26 March and increased between 29 May and 3 June, returning to a concentration similar to those measured during the summer.

5.3.3. Vegetative storage protein abundance

In each water regime, seasonal changes in VSP abundance were similar among the four cultivars with no interaction effect between genotypes and sampling date detected. Western blotting confirmed that the bands selected on the SDS-PAGE gels for quantification corresponded to lucerne taproot VSPs (Figures. 5.4 and 5.5). Under dryland conditions, the abundance of all three VSPs increased between 8 January and 10 March, declined between 10 March and 29 April, and increased again between 29 April and 3 June (Table 5.4). Under irrigated conditions, the abundance of the three VSPs decreased between 26 November and 26 March (Table 5.4). The abundance of the low and high molecular weight VSPs increased between 29 April and 3 June (Table 5.4). Under irrigated conditions, taproot VSP accumulation in June 2008 did not increase to levels measured in the previous November.

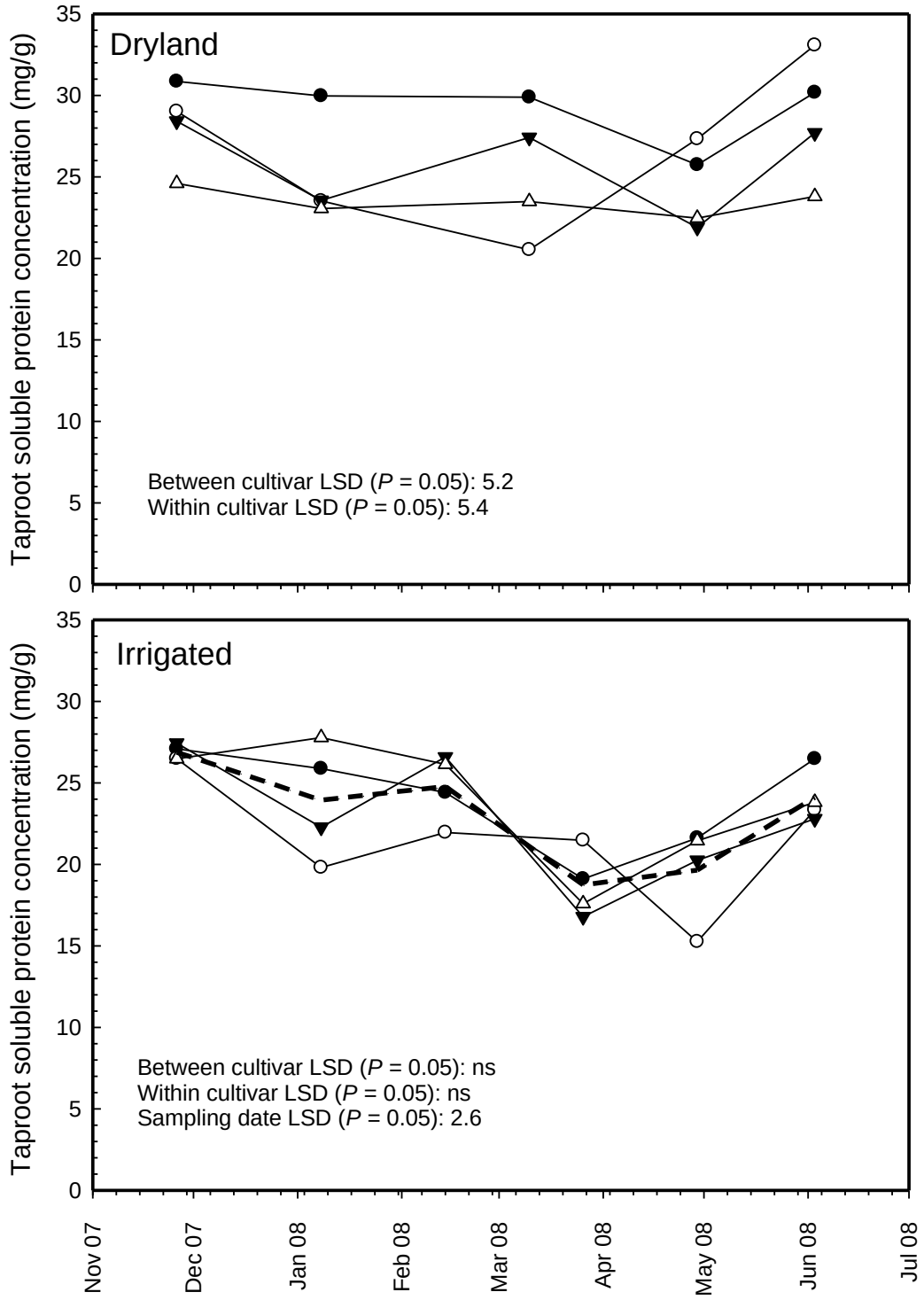

Figure 5.3. Taproot soluble protein concentrations from November 2007 to June 2008 of four lucerne cultivars (DuPuits: ○; Grassland Kaituna: ●; SARDI 7 ▼; SARDI 10: △) grown under dryland or irrigated conditions at Elliott, Tasmania. The broken line represents the average soluble protein concentration for all cultivars grown under irrigated conditions.

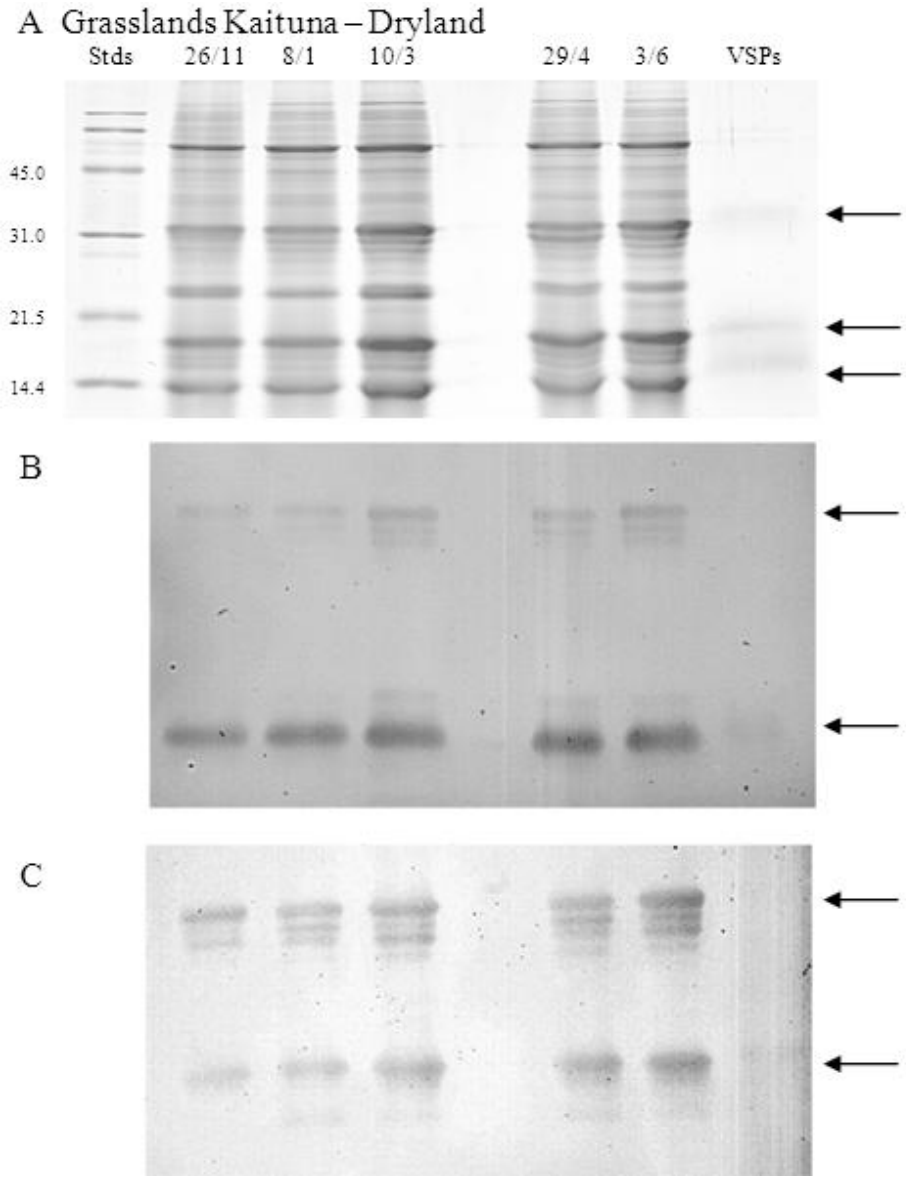

Figure 5.4. SDS-PAGE analysis of the taproot soluble protein pool (A) and corresponding Western blots with anti-bodies raised to the low (B) and middle (C) molecular weight vegetative storage proteins (VSPs) in Grasslands Kaituna lucerne grown under dryland conditions at Elliott, Tasmania and sampled over the 2007/08 season. The far left lane of the gel was loaded with molecular weight standards (Stds), and the numerals represent their sizes in kD. The far right lane of the gel and Western blots were loaded with purified lucerne VSPs, and the arrows on the right highlight their 15, 19 and 32 kD sizes. Both Western blots show the cross antigenicity of the anti-bodies made to the low and middle molecular weight VSPs with the high molecular weight VSP.

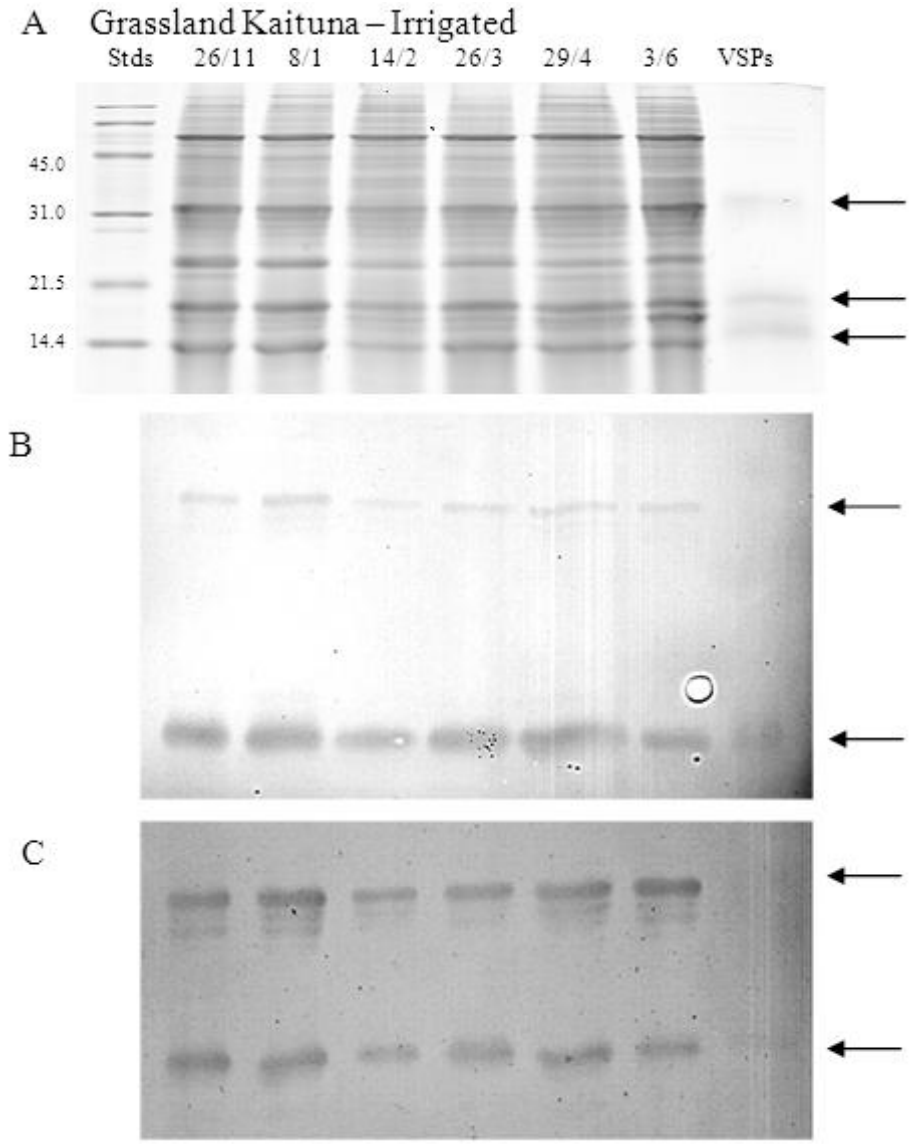

Figure 5.5. SDS-PAGE analysis of the taproot soluble protein pool (A) and corresponding Western blots with anti-bodies raised to the low (B) and middle (C) molecular weight vegetative storage proteins (VSPs) in Grasslands Kaituna lucerne grown under irrigated conditions at Elliott, Tasmania and sampled over the 2007/08 season. The far left lane of the gel was loaded with molecular weight standards (Stds), and the numerals represent their sizes in kD. The far right lane of the gel and Western blots were loaded with purified lucerne VSPs, and the arrows on the right highlight their 15, 19 and 32 kD sizes. Both Western blots show the cross antigenicity of the anti-bodies made to the low and middle molecular weight VSPs with the high molecular weight VSP.

Table 5.4. The differences in optical intensities between the 26 November 2007 sampling and later sampling dates of bands from SDS-PAGE gels that correspond to the high molecular weight (HMW), middle molecular weight (MMW) and low molecular weight (LMW) vegetative storage proteins (VSPs) from lucerne taproots grown at Elliott, Tasmania. Values averaged over four cultivars.

	HMW VSP	MMW VSP	LMW VSP
Sampling date		Dryland	
	Arbitrary units of optical intensity		
26/11/2007	1034	1195	1008
	Difference in optical intensity, % of 26/11/2007		
26/11/2007	0	0	0
08/01/2008	-3	-5	-3
10/03/2008	11	18	25
29/04/2008	-10	-3	0
03/06/2008	13	17	51
LSD (P = 0.05)	15	12	22
Sampling date		Irrigated	
	Arbitrary units of optical intensity		
26/11/2007	983	1169	1144
	Difference in optical intensity, % of 26/11/2007		
26/11/2007	0	0	0
08/01/2008	-7	-12	-12
14/02/2008	-17	-15	-16
26/03/2008	-19	-29	-37
29/04/2008	-30	-32	-31
03/06/2008	-13	-22	-15
LSD (P = 0.05)	17	14	16

5.3.4. Gene transcript abundance

Under dryland conditions there was no effect of cultivar or sampling date on the abundance of RNA transcripts encoding for any of the genes of interest in autumn (Figure 5.6; Table 5.5). All RNA transcripts were present in the taproots (Figure. 7), and there was neither an increase nor a decrease in abundance in any cultivar between the 29 April and the 3 June samplings.

Under irrigated conditions the abundance of RNA transcripts encoding the cold acclimation genes CAR1, Cas17/18 and Cas18 was greater in taproots sampled on 29 April when compared to 3 June (Figure 5.6; Table 5.5). CAR1 and Cas18 were also more abundant in DuPuits taproots when compared to those of SARDI 7 and SARDI 10 (Table 5.5). For Cas18 the abundance of transcripts in taproots of Grasslands Kaituna was greater than SARDI 10. The transcript abundance for β-amylase followed a similar pattern to that of CAR1. This is contrary to what was seen with the VSP transcript levels. When plants were irrigated over summer, the high molecular weight VSP transcript levels increased in taproots of all cultivars between the 29 April and 3 June sampling dates. The transcript abundance of sucrose synthase was not affected by cultivar or sampling date under either water regime.

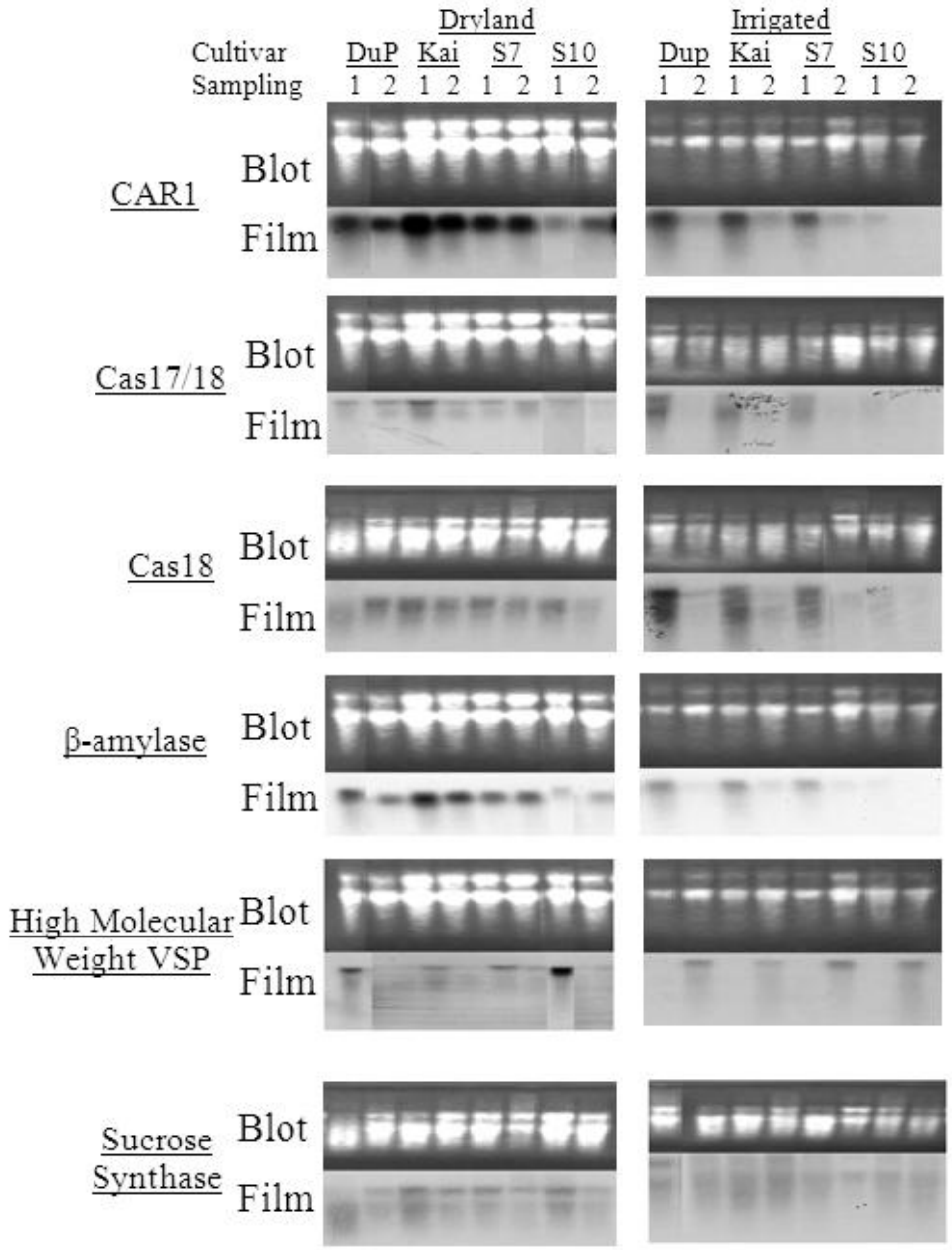

Figure 5.6. Northern analysis of the abundance of RNA transcripts of genes of interest (both RNA blots and films) created from RNA extracted from taproots of lucerne cultivars, DuPuits (DuP), Grasslands Kaituna (Kai), SARDI 7 (S7) and SARDI 10 (S10) grown under dryland or irrigated conditions at Elliott, Tasmania, and sampled on 29 April (1) and 3 June 2008 (2). Blots were probed with radio-labelled cDNA corresponding to the lucerne genes CAR1, Cas17/18, Cas18, β-amylase, high molecular weight vegetative storage protein (VSP), and sucrose synthase.

Table 5.5. Normalised optical intensity of lanes from Northern blots corresponding to four lucerne cultivars, sampled on 29 April and 3 June 2008, that were grown with or without irrigation at Elliott, Tasmania. Blots were probed with radio-labelled cDNA corresponding to the lucerne genes CAR1, Cas17/18, Cas18, β-amylase, high molecular weight vegetative storage protein (HMW VSP), and sucrose synthase.

	CAR1	Cas17/18	Cas18	β-amylase	HMW VSP	Sucrose synthase
Cultivar			Dryland			
DuPuits	35	20	36	13	18	21
Grasslands Kaituna	30	10	16	12	13	16
SARDI 7	19	15	16	8	27	11
SARDI 10	21	11	24	8	25	14
LSD (P = 0.05)	†ns	ns	ns	ns	ns	ns
Harvest date						
29/04/2009	28	15	26	11	26	18
03/06/2009	25	13	20	9	15	12
LSD (P = 0.05)	ns	ns	ns	ns	ns	ns
Cultivar			Irrigated			
DuPuits	48	18	36	19	30	17
Grasslands Kaituna	40	21	30	16	35	18
SARDI 7	20	8	18	8	66	15
SARDI 10	14	12	12	6	64	14
LSD (P = 0.05)	27	ns	17	11	ns	ns
Harvest date						
29/04/2009	47	20	34	19	32	18
03/06/2009	15	10	14	6	66	14
LSD (P = 0.05)	22	8	15	9	23	ns

†ns: no significant effect

5.4. Discussion

Summer irrigation and cultivar interacted to affect taproot carbohydrate and N pools. However, this interaction was not consistent between the two pools. Cultivar by season interactions on changes in taproot protein concentration were only exhibited under dryland conditions (Figure 5.3). Soluble protein concentrations increase in winter dormant cultivars in autumn, while winter active cultivars require exposure to freezing temperatures before accumulation begins (Cunningham *et al.* 1998). While the autumn increase in taproot soluble protein concentration was observed for DuPuits grown under dryland conditions, other cultivars maintained stable taproot soluble protein concentrations from summer into autumn (Figure 5.3). The absence of freezing temperatures in this environment (Figure 4.1) would have prevented the more winter active cultivars from showing an increase in soluble protein concentrations. Previous studies comparing seasonal changes in taproot soluble protein concentrations (Cunningham *et al.* 1998) in genotypes with differing levels of winter activity, have been undertaken under rainfed conditions. Under irrigated conditions taproot protein concentrations in all cultivars declined over summer before increasing in autumn. The use of taproot reserves is controlled by shoot growth, so, as all cultivars maintained high growth rates when irrigated (Chapter 4), N reserves were utilised to maintain shoot growth and cultivar differences in taproot soluble protein concentrations were negated.

The relative abundance of VSPs in taproots of plants grown with irrigation followed the seasonal decrease and increase in soluble protein concentrations, while under dryland conditions VSP abundance increased during summer in all cultivars (Table 5.4). The decrease in the amount of VSP over summer in plants grown under irrigated conditions can be attributed to herbage growth throughout the growing season, as VSPs have been identified as the primary N source for the production of new N-rich tissues after defoliation (Avice *et al.* 1997b). An increase in VSP abundance in plants grown under drought stress has been observed in glasshouse experiments (Erice *et al.* 2007), but not under field conditions (Justes *et al.* 2002). In the experiment reported by Justes *et al.* (2002), plants were establishing and had not been defoliated, making a direct comparison of results between the current and their

experiments difficult. The increase in VSP abundance during drought has been proposed to be either a means of salvaging N from stressed shoots or a functional role in drought stress tolerance (Erice *et al.* 2007). While these field results have confirmed glasshouse results showing an increase in VSPs in drought-stressed lucerne that is defoliated, it does not prove nor disprove the possible physiological roles or mechanisms for these proteins proposed by Erice *et al.* (2007).

Contrasting water regimes did not show an overarching effect on the seasonal changes in taproot carbohydrate constituents (Table 5.3). Under both water regimes, throughout the season, starch concentrations decreased while sugar concentrations increased. This contradicts previous reports in un-defoliated seed crops, where taproot TNC increased in those grown under drought stress (Cohen *et al.* 1972). However, in plants grown under glasshouse conditions where defoliation was included with a water deficit, no increase in taproot starch or sugar occurred (Erice *et al.* 2007).

While there was some interaction between irrigation and genotype on both the phenological and molecular expression of winter dormancy, water regime had the greater influence. Water regime had an over-arching effect on sward height in autumn, with the most winter active cultivar grown under dryland conditions having a similar sward height to that of the most winter dormant cultivar grown under irrigated conditions (Figure 5.2). The visual phenotype of winter dormancy (sward height in late autumn; Teuber *et al.* 1998) was seen in plants grown under each water regime, as the more winter active cultivars (SARDI 7 and SARDI 10) grew more than the less winter active cultivars (Grasslands Kaituna and DuPuits; Figure 5.2). The reversal in sward height between Grasslands Kaituna and DuPuits in plants grown under irrigation, when compared to dryland conditions was un-expected (Figure 5.2). However, plasticity in the winter activity rating of Grasslands Kaituna across differing environments in Tasmania has been previously observed (R. S. Smith pers. comm. 2006), and a genotype by environment interaction on the expression of winter dormancy in lucerne grown in California has been reported by Teuber *et al.* (1998).

Apart from sucrose synthase, transcript profiles of all genes investigated were altered by irrigation. Even though the concentration of soluble sugar did not increase between April and June (Table 5.6), sugar concentrations were already at high levels in plants grown under either water regime, therefore the absence of changes in sucrose synthase transcript abundance was expected. The abundance of transcripts encoding the high molecular weight VSP was only enhanced in plants grown under irrigated conditions (Table 5.5). VSP abundance did not change in plants that were grown under dryland conditions. These plants grew less than irrigated plants in summer, and consequently they did not utilise their N reserves, reducing the need to increase VSP abundance in autumn as part of the cold acclimation process. While no measurement of β-amylase abundance was made in this study, it has been postulated that β-amylase has a role as a VSP in lucerne taproots and is responsive to the same environmental stimuli that increases the other lucerne taproot VSPs (Gana *et al.* 1998). An increase in β-amylase transcript abundance between April and June in irrigated plants was not observed. Under irrigated conditions the β-amylase transcript behaved like the genes associated with cold acclimation (Table 5.5).

Transcript levels of genes associated with cold acclimation (CAR1, Cas17/18 and Cas18) were elevated in the middle of autumn in plants grown under irrigated conditions, and the CAR1 and Cas18 genes also showed cultivar effects. Increased transcript levels of cold acclimation genes in plants with declining winter activity ratings has been observed by Haagenson *et al.* (2003a) and Pennycooke *et al.* (2008), and this effect was observed for CAR1 and Cas18 in this field experiment. The consistent high levels of expression of the cold acclimation genes in plants grown under dryland conditions in late autumn raises questions about their specific function and whether the expression of cold acclimation genes is a casual effect of winter dormancy. This question has been raised previously by Haagenson *et al.* (2003a) and Wolfraim *et al.* (1993). Both Cas17 and Cas18 share amino acid homology to motifs of proteins belonging to the dehydrin family and are thought to help protect cells during dehydration associated with freezing, while CAR1 shares nucleotide sequence homology to Cas15b (Genbank accession L12462; Haagenson

et al. 2003a), a punitive nuclear signalling protein (Monroy *et al.* 1993). While no data on the expression of these genes under drought over the summer is available, their punitive dehydration-related function may result in higher RNA transcript levels over summer under dryland conditions and this expression being maintained into winter.

Metochis and Orphanos (1981) observed that lucerne crops, following release from severe summer-long drought, maintained a dormant phenotype until they were defoliated. In the current experiment, lucerne was not defoliated after the summer drought was broken by autumn rains. Shoots are the plant organ with control over the expression of winter dormancy (Heichel and Henjum 1990). Results of Metochis and Orphanos (1981) suggest that shoots may also exhibit control over drought dormancy. If winter-dormancy genes are also expressed as a drought response, and plants were not defoliated, their expression may have been maintained despite the removal of the drought stress, as was observed in the current study.

Irrigation and genotype interacted to affect the soluble protein reserve pool, VSP accumulation and the expression of key gene transcripts influencing plant N reserve storage and winter dormancy. The influence of the soluble protein pool on shoot DM accumulation as documented in the literature suggests that the impact of water deficit on yield is at least partially associated with alterations in this reserve pool. The influence that water deficit has on the expression of winter dormancy genes may explain the improved performance of the more winter dormant genotypes under dryland conditions in Chapter 4. Further studies are now required to identify other factors apart from the taproot reserve pools that may be affecting shoot growth and to investigate the role of winter dormancy genes in drought adaptation.

CHAPTER 6

The effect of water deficit on canopy structure and photosynthesis during the regrowth of lucerne

6.1. Introduction

In the cool temperate regions of Australia low rainfall and high daily evaporation experienced over the summer month's results in severe soil moisture deficit conditions. The effect of moisture stress on the growth and canopy structure of lucerne has been well studied Lucerne has a global distribution across environments ranging from 250 to 1500 mm annual rainfall (Brown and Tanner 1983;Carter and Sheaffer 1983a; Durand *et al.* 1989). However, these authors only investigated one genotype. Any environmental influence on canopy structure of lucerne is mediated by genotype (Frame *et al.* 1998). Genotypic influences however, are not strongly displayed under field conditions in cool temperate climates (Chapter 4), as high plant densities and associated interplant competition in lucerne crops often prevent genotypes with the potential to produce higher shoots per plant from expressing this trait (Durand *et al.* 1989). The previous study of yield components under field conditions found that shoots per plant are affected by genotype only in a low rainfall environment (Chapter 4). As yet no investigation of a genotype by water deficit on other factors affecting shoot growth and plant DM accumulation for contrasting lucerne genotypes during the regrowth period has been undertaken.

Identifying if, and to what extent, the photosynthetic apparatus is damaged and carbon fixation capacity is reduced by water deficit is important as this is the major source of carbon for regrowing shoots post defoliation. Past authors have noted that changes in canopy characteristics (both structure and capacity for photosynthesis) affect growth during and after a water deficit (Metochis and Orphanos 1981; Halim *et al.* 1989; Frame *et al.* 1998) This chapter will examine the hypothesis that the severity of water deficit determines the response of shoot

development and plant photosynthetic capacity to water deficit. The objective was to investigate the impact of differing water deficits during a regrowth cycle on canopy structure and photosynthetic capacity of two lucerne cultivars identified as having contrasting performance under dryland and irrigated conditions.

6.2. Materials and Methods

6.2.1. Growing conditions and experimental design

Plants were grown in the greenhouse facility of the Tasmanian Institute of Agricultural Research in Burnie, Tasmania (41^{o}04'S, 145^{o}53'E; elevation 206 m), for the duration of the experiment. A greenhouse environment control system (Priva Maximizer, Priva Computers Inc, Vineland Station, Ontario, Canada) was used to maintain canopy temperatures at 21 ± 2°C during the day and 11 ± 2°C at night. These temperatures are representative of summer temperatures experienced in the cool temperate dairy regions of Australia (Figure 2.1). Relative humidity ranged between 21 to 91%, while photosynthetic photon flux density between 1000 and 1600 hours ranged between 100 and 1150 μmol m/s. Three 400 W halogen bulbs suspended 2 m above the plants were used to maintain a photoperiod of 14 hours with a 30 minute transition between full light and dark conditions. Daily evaporation averaged 2.25 ± 0.08 mm. Temperature, relative humidity and photosynthetic active radiation at the canopy were monitored every 10 min using automated sensors and a data logger (HOBO micro station, Onset Computer Corporation), while evaporation was measured daily with a US Class A pan.

Two cultivars, Grasslands Kaituna (semi winter-dormant) and SARDI 10 (highly winter-active) were grown in 4.5 L polyethylene bag pots with drainage holes. The growing medium (5.0 kg per pot) was a sandy loam soil sourced from a local landscape supplier. At a soil water potential of -10 kPa the soil volumetric water content was 20% (determined with theta probes; Delta-T Devices Ltd. Cambridge, UK and gypsum blocks; Irrometer. Riverside CA, USA). Prior to the addition of fertilizer, soil fertility was 4.9 mg/kg P (Olsen extraction) and 49 mg/kg K (Colwell extraction) with a $pH_{(water)}$ of 5.6. Based on these values, 0.21 g of P (molybdenum

fortified single super phosphate), 0.38 g of K (muriate of potash) and 3.4 g of lime per kg of soil were added to bring soil chemical characteristics into the optimum ranges for lucerne growth.

Plants were grown until 64 days after emergence (DAE), at which time they were defoliated to 50 mm height. Herbage was dried at 60^{o}C for 48 hours and weighed. Pots with herbage yields greater than one standard deviation above or below the mean of all pots were removed from the experiment. The remaining plants were then grown for a further 44 days (108 DAE) until crown bud elongation was apparent. Up until 108 DAE, all pots received 100% of the replacement water requirement. At this time, plants were defoliated to 50 mm and the deficit watering treatments began (from here on referred to as H_0). Five levels of water deficit were created by applying 100, 75, 50, 25 and 0% of the replacement water requirement to return volumetric SWC to 20% (soil water potential of -10 kPa). Plants were destructively harvested immediately before initiation of the water deficits (H_0) and following H_0, at seven day intervals for 35 days. The total number of pots in the experiment was 240 (four replications, two cultivars, five levels of water deficit and six destructive samplings). The experiment consisted of four replications blocked based on greenhouse benches. Pots were re-randomised within each block every seven days to reduce within block variation. A buffer of fully watered pots of lucerne, that were regularly trimmed to the height of adjacent experimental plants, surrounded the outer borders of the blocks.

6.2.2. Planting, establishment and water delivery

A uniform soil bulk density in each pot was created by placing 2.5 kg of soil into each pot and a 6.0 kg weight was placed on the soil surface for 10 seconds. The remainder of the soil was then added to each pot (2.5kg/ pot) and the 6.0 kg weight placed back on the soil surface for a further 10 seconds. Finally the pot was irrigated to saturation and allowed to drain for 62 hours. Uncoated seed of each cultivar was sown into moist soil at a depth of 10 mm. When the first true leaf emerged after germination, seedlings were spray inoculated with *Rhizobia* (Group AL; Beecker Underwood Pty Ltd.) and immediately watered by hand to wash the inoculant into the soil. When an average of three true leaves per plant had emerged, plants were

thinned to eight plants per pot. From this time onwards a drip irrigation system was used to deliver water to the pots.

Water was delivered to the pots using pressure-compensating drip emitters (2.00 ± 0.02 L/h) anchored to the soil surface in the centre of each pot. Pressure through the irrigation system was regulated at 138 kPa. The amount of water to return the pots to a soil volumetric water content of 20% (soil water potential of –10 kPa) was calculated daily by monitoring the soil water content (Theta probes) of four pots of lucerne receiving 100% of the replacement water requirement. The water required for each treatment was delivered on a timed basis.

6.2.3. Measurements and sampling

6.2.3.1. Plant tissue sampling

At each destructive sampling all herbage above a height of 50 mm was defoliated. The defoliated herbage was then weighed, mixed and split into two sub-samples of equal mass. The total number of shoots in one sub-sample was counted, and then hand-separated into leaf and stem. The shoots in the other sub-sample were grouped based on morphological maturity stage (Kalu and Fick 1981) and counted. All separated samples were dried at 60^{o}C for 48 hours and weighed. Herbage DM yield per plant, shoot mass, number of shoots per plant, MSC, MSW and leaf-to-stem ratio were calculated.

The number of shoots arising from the crown and the number of shoots arising from nodes on another shoot (axial shoots) were counted on the remaining herbage below the 50 mm cutting height and crowns. The ratio of axial to crown shoots was calculated.

6.2.3.2. Shoot water measurements

Shoot water potential was determined before H_0, and then at 8, 18, 23 and 30 days after H_0. A pot of each cultivar from each treatment by block combination was randomly selected, and the upper 50 mm of two shoots were severed, immediately wrapped in plastic film and placed on ice as described by Ward and Micin (2006).

Shoot water potential was determined by using a pressure chamber (PMS model 615, PMS instrument company, Albany, Oregon, USA). All measurements of shoot water potential were undertaken between 0800 and 1000 hours, and were assessed within one hour of the shoot being severed.

Estimated ET was determined 5, 10, 18, 24 and 33 days after H_0. Predetermined pots of both cultivars from each treatment by block combination were weighed. Twenty-four hours later the same pots were weighed again. At this time the canopy height of each pot was measured. Estimated ET and ET adjusted using plant height (relative ET) were calculated from the soil surface area and mass difference of the pot over the 24 hour period.

6.2.3.3. Physiological measurements of plants

Measurements of chlorophyll fluorescence occurred before H_0, and then at 10, 15, 27 and 34 days after H_0. Parameters were measured using a portable fluorometer (Mini-PAM, Heinze Walz GmbH Effeltrich, Germany). All measurements were undertaken at 20±3^{o}C using the saturation pulse method described in the Mini-PAM manual (Heinze Walz GmbH 1999). Prior to measurements, plants were dark-adapted for 30 minutes. For both cultivars from each treatment the maximum efficiency of photosystem II (Fv/Fm) for the middle leaflet of every leaf on two shoots was determined. Care was taken to avoid the leaflet midrib. Measurements were undertaken on the same shoots at each sampling time.

Gas exchange was measured with an infra-red gas analysis system (LCi portable photosynthesis system, ADC BioScientific, Hoddesdon, UK). Measurements were undertaken between 1000 and 1400 hours, at 9, 19, 24 and 33 days after H_0. The third highest leaf on two predetermined shoots of each cultivar per treatment by block combination was measured. The same shoots were used for measurements at each assessment. Ambient air CO_2 concentration was measured between 365 and 370 µg/L. Carbon dioxide exchange rate, transpiration rate and stomatal conductance to water vapour were measured. Digital photos were taken of each leaf in the leaf chamber at the time of measurement. These images were analysed with image analysis software (IMAGE J, National Institute of Health) to

determine the leaf area being measured. Parameters were adjusted to account for leaf area.

6.2.4. Statistical analysis

Data gathered from the destructive harvests were analysed using ANOVA of a randomised complete block design. Mean stage count, MSW, leaf-to-stem ratio, and axial to crown shoot ratio data were transformed using the square root transformation as for these data, the variance was proportional to the treatment mean. Quadratic regressions were fitted to the DM accumulation data. Shoot water potential was analysed using ANOVA of a randomised complete block design. Plant height, canopy water use, and gas exchange parameters were analysed using ANOVA of a blocked split plot in time design. The Fm/Fv ratio of the oldest leaf on the shoot, youngest leaf on the shoot, and the average Fv/Fm ratio of all leaves on the shoot were analysed using ANOVA of blocked split-plot-in-time designs. At each measurement linear regressions of the Fm/Fv ratio against leaf position on the shoot (from bottom to top) were developed for each cultivar by treatment combination.

Where ANOVA identified a significant effect, Fishers LSD was used to identify differences between treatments. Except where otherwise stated, significance was accepted when $P < 0.05$. All analyses were undertaken using Genstat 9.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK).

6.3. Results

6.3.1. Yield components and dry matter accumulation

Water deficit treatments had a significant effect on both number of shoots per plant and shoot mass, resulting in reduced biomass accumulation (Table 6.1). The number of shoots on plants grown under a severe water deficit (25% or 0% of the replacement water requirement) peaked 14 days after H_0. In comparison, plants receiving either 100%, 75% or 50% of the replacement water requirement maintained the development of new shoots for a longer period, attaining maximum shoot number 21 days after H_0.

Mass per shoot increased throughout the 35 days of regrowth for all but the plants receiving 25% or 0% of the replacement water requirement (Table 6.1). The rate of increase in mass per shoot in the plants growing with 75% or 50% of the replacement water requirement was lower than the plants receiving 100% of the replacement water requirement. At 35 days after H_0, differences in shoot mass reflected the level of water deficit the plants were exposed to. For example, the mass per shoot of plants receiving 75%, 50% and 25% of the replacement water requirement was 71%, 51% and 36% respectively, of the plants receiving 100% of the replacement water requirement.

The ratio of axial to crown shoots decreased as the plants regrew ($P < 0.01$), with this ratio dropping from an average of 0.75:1 at 7 days to 0.40:1 at 21 days after H_0. Lack of water reduced the proportion of axial shoots from an average of 0.51:1 for the plants receiving 100% water to 0.09:1 for the plants receiving no water. Across all treatments Grasslands Kaituna averaged a larger proportion of axial shoots per plant than SARDI 10 (average axial to crown shoot ratio of 0.58:1 vs. 0.48:1).

The negative impact of water deficit on both shoot mass and shoot number combined to reduce herbage DM accumulation and growth rate ($P < 0.001$). Apart from the plants receiving no water, quadratic regressions of DM accumulation against days of regrowth were significant for all levels of water deficit (Figure 6.1). Regressions showed that the rate of DM accumulation was highest in plants receiving 100% of the replacement water requirements. A plot of the regressions (Figure 6.1) showed that the plants receiving 25% of the replacement water requirement had reached maximum yield by 21 days of regrowth. The rate of DM accumulation of the plants receiving 75% and 50% of the replacement water requirement decreased over time, while the growth rates of the unstressed plants continued to increase through the 35 days of regrowth.

Table 6.1. The yield components (shoots per plant and mass per shoot) of lucerne (cultivars Grassland Kaituna and SARDI 10 averaged at each level of water deficit) regrowing under five different levels of water deficit; 100, 75, 50, 25 and 0% of the replacement water requirement applied.

	Days after H_0†					
Treatment	Pre-defoliation	7	14	21	28	35
	Shoots per plant					
100% water	2.36	1.53	2.55	3.39	3.13	3.24
75% water		1.09	2.25	2.88	2.48	2.85
50% water		0.83	1.82	2.23	2.28	2.23
25% water		0.89	1.57	1.32	1.12	1.42
0% water		0.25	0.25	0.38	0.31	0.00
LSD (P = 0.05)				0.32		
Grasslands Kaituna	2.55	0.81	1.65	2.12	1.78	1.96
SARDI 10	2.17	1.02	1.72	1.95	1.93	1.94
LSD (P = 0.05)				ns‡		
	Mass per live shoot (mg/shoot)					
100% water	132	69	84	119	120	215
75% water		64	84	94	128	153
50% water		70	80	80	131	115
25% water		77	85	82	111	79
0% water		56	22	0	0	0
LSD (P = 0.05)				30		
Grasslands Kaituna	120	61	74	75	93	115
SARDI 10	145	74	67	75	103	110
LSD (P = 0.05)				ns		

H_0†: second defoliation (108 DAE)
ns‡: no significant effect

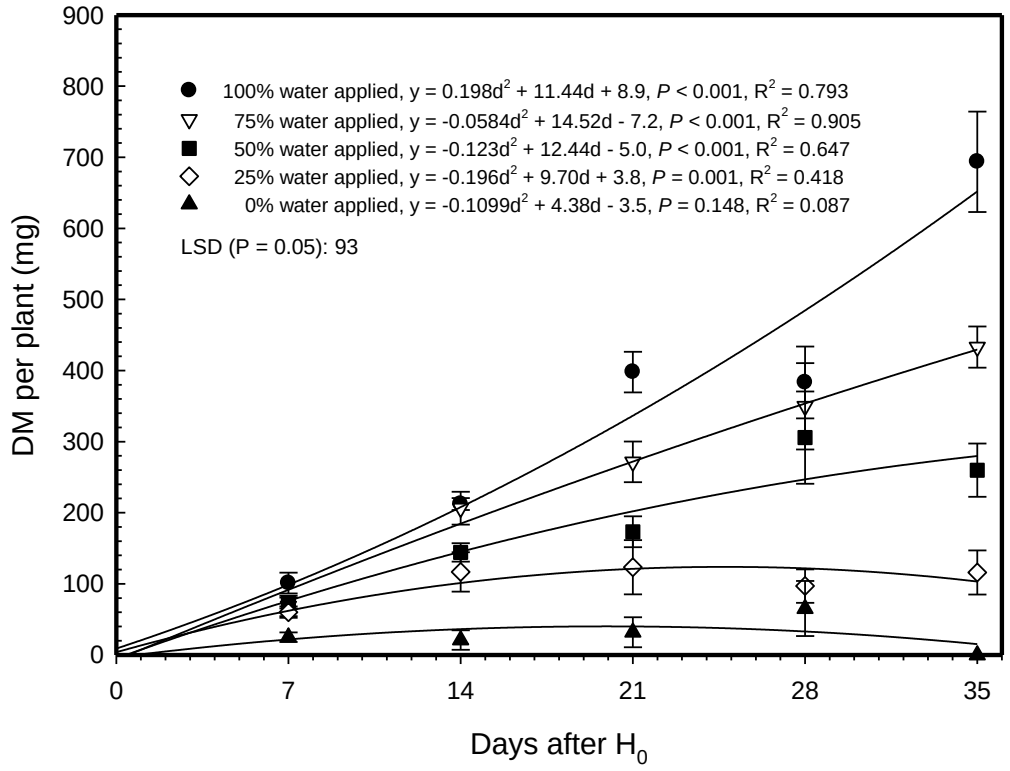

Figure 6.1. Regressions of dry matter (DM) accumulation per plant of lucerne (cultivars Grassland Kaituna and SARDI 10 averaged at each level of water deficit) regrowing after defoliation (H_0) under various levels of water deficit; 100, 75, 50, 25 and 0% of the replacement water requirement. Error bars represent the standard errors of the means.

6.3.2. Morphological maturity and leaf to stem ratio

At 14 days after H_0, water deficit began to slow plant morphological development. At this time MSC and MSW were greater in fully-watered plants compared to the plants regrowing under a water deficit (Table 6.2). While the fully-watered plants were more mature than plants under a water deficit, both MSC and MSW still classified the plants within the early and mid-vegetative stages, reflecting the high number of vegetative shoots present on the plants even after 35 days of regrowth. Increases in MSC and MSW were delayed until 28 days of regrowth in the plants receiving 75 and 50% of the replacement water requirement, while plants receiving only 25% of the replacement water requirement showed no signs of an increase in these parameters. There was a consistent influence of cultivar on plant MSC and MSW throughout the experiment, with SARDI 10 being more mature than Grasslands Kaituna ($P < 0.01$).

The leaf-to-stem ratio of the plants receiving 100% and 75% of the replacement water requirement remained consistent throughout the 35 days of regrowth. By comparison, the plants that received only 50% or 25% of the replacement water requirement displayed an increase in the leaf-to-stem ratio by day 14 of regrowth (Table 6.2). This resulted in the plants under the greater moisture deficits producing a more compact canopy structure. Both cultivars maintained similar leaf-to-stem ratios, and, for the 35 days after H_0, averaged 2.5:1 for Grasslands Kaituna and 2:1 for SARDI 10.

Gas exchange and chlorophyll fluorescence parameters

Leaf death on the plants receiving no water resulted in a reduction or elimination of CO_2 exchange and transpiration rates (Figure 6.2). Plants that were exposed to other levels of deficit watering maintained rates similar to those of plants receiving 100% of the replacement water requirement, except at 19 days after H_0. At this time, plants receiving 25% of the replacement water requirement had lower CO_2 exchange and transpiration rates ($P < 0.05$). There was no significant difference in the CO_2 exchange rate or transpiration rate between the two cultivars. Despite the limited

impact of water deficit on transpiration rate throughout the regrowth period, at certain times during regrowth (Figure 6.2), deficit levels of water application significantly affected the stomatal conductance to water vapour ratio of the plants.

Maximum efficiency of photosystem II for the average of all leaves on the shoot was affected by an interaction between water deficit and time ($P < 0.001$). The Fv/Fm of the plants receiving no water was less than the other plants by 15 days after H_0. This decrease in Fv/Fm continued as the leaves on the plants receiving no water senesced and the Fv/Fm reached zero (Figure 6.3). The response in Fv/Fm for the youngest and oldest leaves showed the same pattern as the average of all leaves on the shoot (data not shown). Regressions of leaf position against Fv/Fm ratio for each cultivar by treatment combination showed that there was no significant influence from leaf position on the Fv/Fm ratio in either cultivar or level of water deficit.

Table 6.2. The morphological maturity parameters mean stage count (MSC), mean stage weight (MSW), and leaf to stem ratio (L:S) of lucerne (cultivars Grassland Kaituna and SARDI 10 averaged at each level of water deficit) regrowing under different levels of water deficit (100, 75, 50, 25 and 0% of the replacement water requirement applied).

	Days after H_0[†]					
Treatment	Pre-defoliation	7	14	21	28	35
				MSC		
100% water	0.78	0.00	0.20	0.58	0.57	0.70
75% water		0.00	0.23	0.21	0.43	0.45
50% water		0.00	0.07	0.08	0.30	0.29
25% water		0.00	0.06	0.10	0.01	0.00
0% water		0.00	0.00	0.00	0.00	0.00
LSD (P = 0.05)				*0.22*		
Grasslands Kaituna	0.69	0.00	0.06	0.14	0.15	0.22
SARDI 10	0.85	0.00	0.16	0.22	0.38	0.31
LSD (P = 0.05)				ns[‡]		
				MSW		
100% water	1.27	0.00	0.31	0.88	0.96	1.07
75% water		0.00	0.31	0.32	0.74	0.71
50% water		0.00	0.15	0.14	0.41	0.56
25% water		0.00	0.08	0.15	0.03	0.00
0% water		0.00	0.00	0.00	0.00	0.00
LSD (P = 0.05)				0.31		
Grasslands Kaituna	1.01	0.00	0.10	0.24	0.31	0.38
SARDI 10	1.28	0.00	0.24	0.30	0.51	0.46
LSD (P = 0.05)				ns		
				L:S		
100% water	1.81	2.98	2.73	1.87	2.68	1.97
75% water		3.30	2.78	2.95	3.01	2.35
50% water		2.09	4.01	3.36	4.03	3.90
25% water		2.21	3.15	2.75	4.92	4.38
0% water		1.03	0.48	0.00	0.00	0.00
LSD (P = 0.05)				1.33		
Grasslands Kaituna	2.03	2.02	2.92	2.32	3.35	2.42
SARDI 10	1.48	2.51	2.08	1.91	2.04	2.06
LSD (P = 0.05)				0.84		

H_0[†]: second defoliation (108 DAE)
ns[‡]: no significant effect

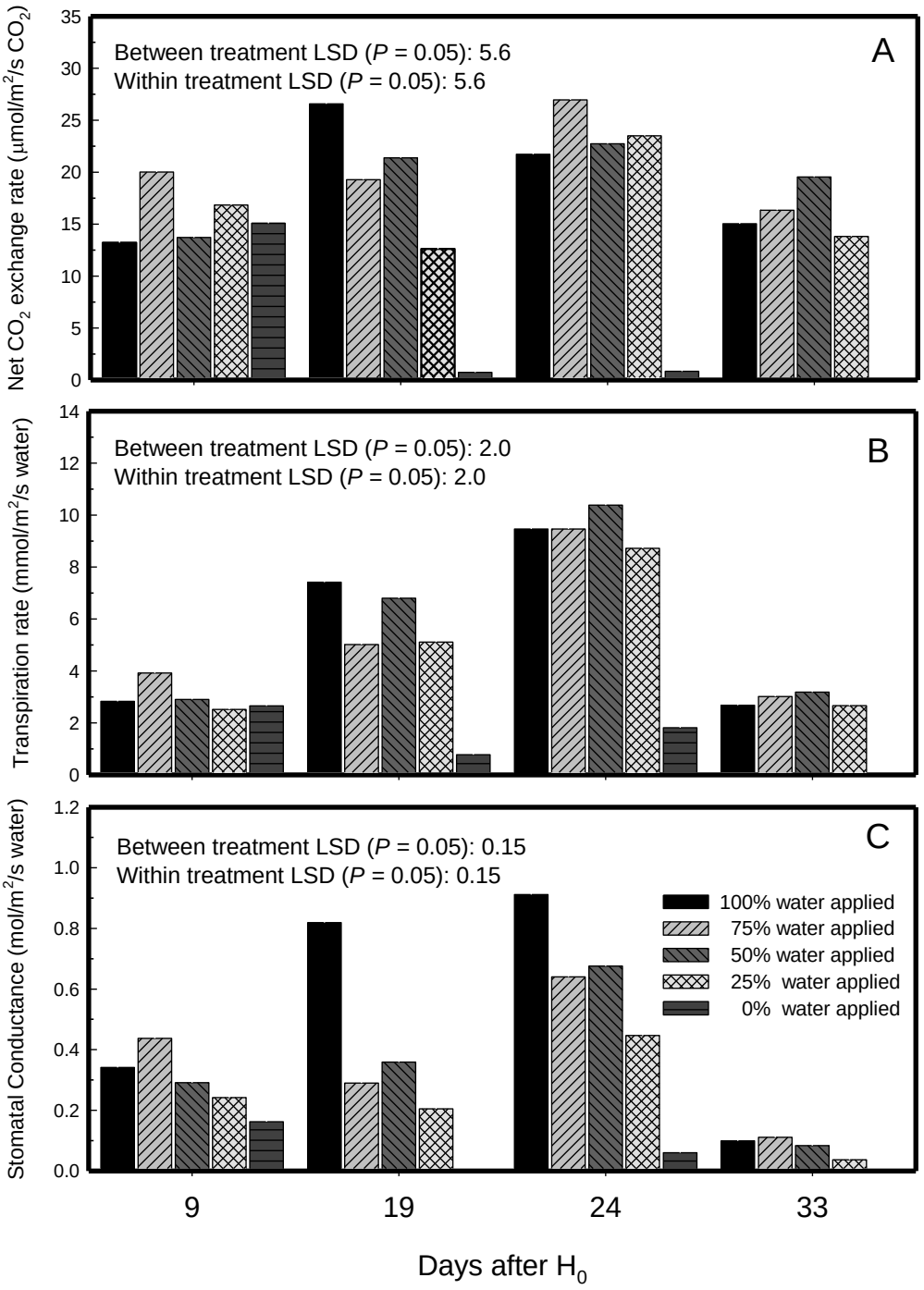

Figure 6.2. Rate of net CO_2 exchange (A), transpiration (B) and stomatal conductance to water vapour (C) of lucerne (cultivars Grassland Kaituna and SARDI 10 averaged at each level of water deficit) regrowing under various levels of water deficit (100, 75, 50, 25 and 0% of the replacement water requirement applied) at 9, 19, 24 and 33 days after H_0.

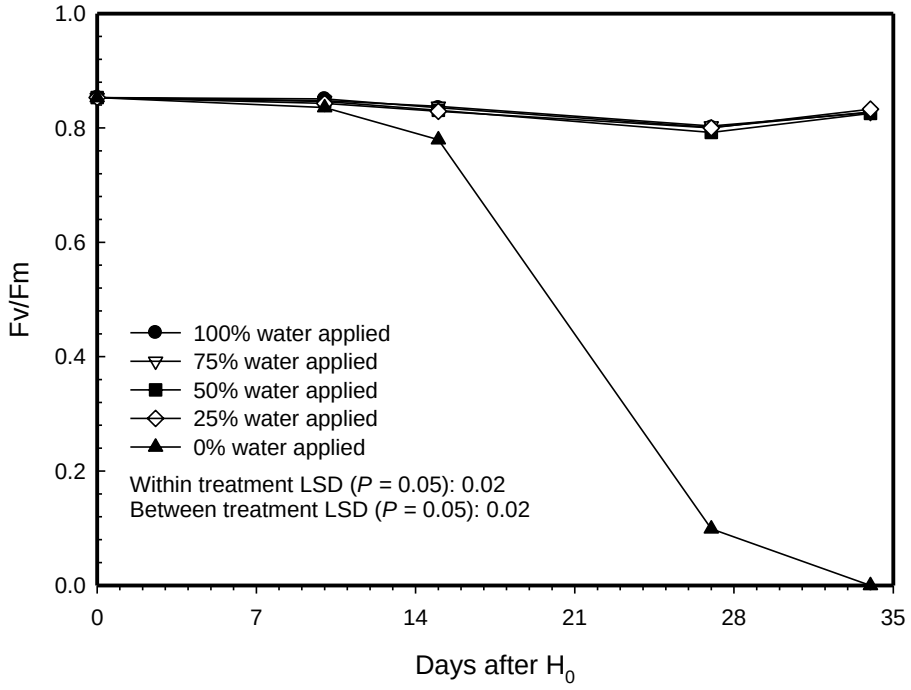

Figure 6.3. Maximum efficiency of photosystem II (Fv/Fm) averaged across all leaves on the shoots of lucerne plants (cultivars Grassland Kaituna and SARDI 10 averaged at each level of water deficit) regrowing under various levels of water deficit (100, 75, 50, 25 and 0% of the replacement water requirement applied).

6.3.3. Shoot water potential

Plants responded to water deficit through a gradual decrease in shoot water potential (Figure 6.4). The extent of the decrease in shoot water potential reflected the level of water deficit to which the plants were exposed. Plants receiving 0% of the replacement water requirement had a rapid decrease in shoot water potential compared to the other water deficit treatments, with plants reaching a shoot water potential of -4.0 MPa 20 days after H_0. Differences between the treatments that received any water were small. Plants that received only 25% of the replacement water requirement had a shoot water potential of -1.88 MPa 30 days after H_0, while plants that received 100% of the replacement water requirement had a shoot water potential of -1.42 MPa. Although the differences were not consistent across treatments, there were differences in shoot water potential between the two cultivars.

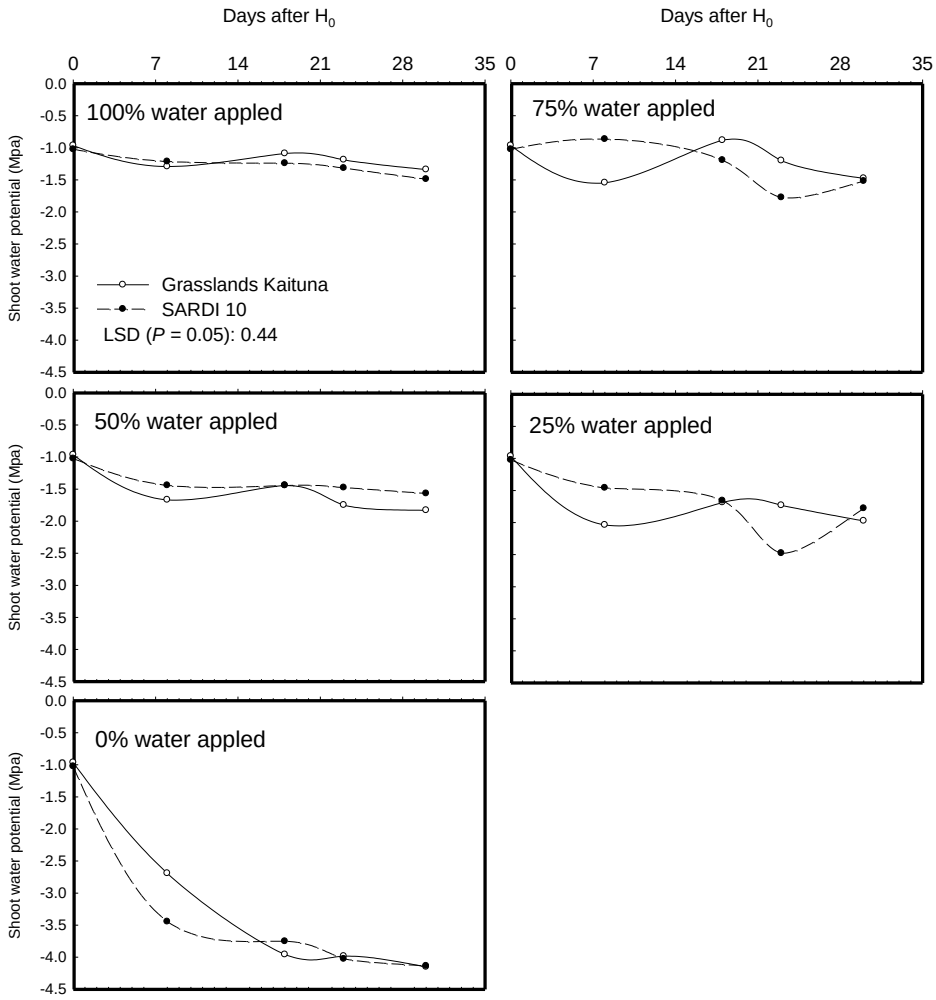

Figure 6.4. Changes in shoot water potential of Grasslands Kaituna and SARDI 10 lucerne regrowing under various levels of water deficit (100, 75, 50, 25 and 0% of the replacement water requirement).

6.3.4. Plant morphology and estimated evapo-transpiration

The level of water deficit significantly ($P < 0.001$) affected plant height. By 10 days of regrowth, segregation in plant height based on the different levels of water deficit was detectable (Figure 6.5). This segregation became more apparent as regrowth continued. Plants regrowing under water deficit produced compact canopies with smaller leaves (Plate 6.1). Shoots and leaves of the plants receiving no water wilted within a week of the initiation of treatments, and shoots began to die by the second week of regrowth. Wilting was not observed on any of the other treatments.

The impact of water deficit on ET and relative ET changed throughout the regrowth period (Figure 6.5). After 18 days of regrowth, plants receiving 100% of the replacement water requirement were using more water per day than plants receiving 50%, 25% or 0% of the replacement water requirement. After 33 days of regrowth, plants receiving 25% or 0% of the replacement water requirement had lower rates of ET than all other treatments.

When plant height was used as an indirect measure of canopy volume, differences in relative ET were detectable after 10 days of regrowth (Fig. 5). Plants regrowing under the greater water deficits (0%, 25% and 50% of the replacement water requirement) used more water per mm of plant height than the less-stressed treatments (75% and 100% of the replacement water requirement). However, after 18 days of regrowth, these differences in relative ET had disappeared, and values remained similar for the remainder of the regrowth cycle.

0% of replacement requirement applied	25% of replacement requirement applied	50% of replacement requirement applied	75% of replacement requirement applied	100% of replacement requirement applied

Plate 6.1. Impact of different levels of water deficit on lucerne canopy development at 21 days after H_0.

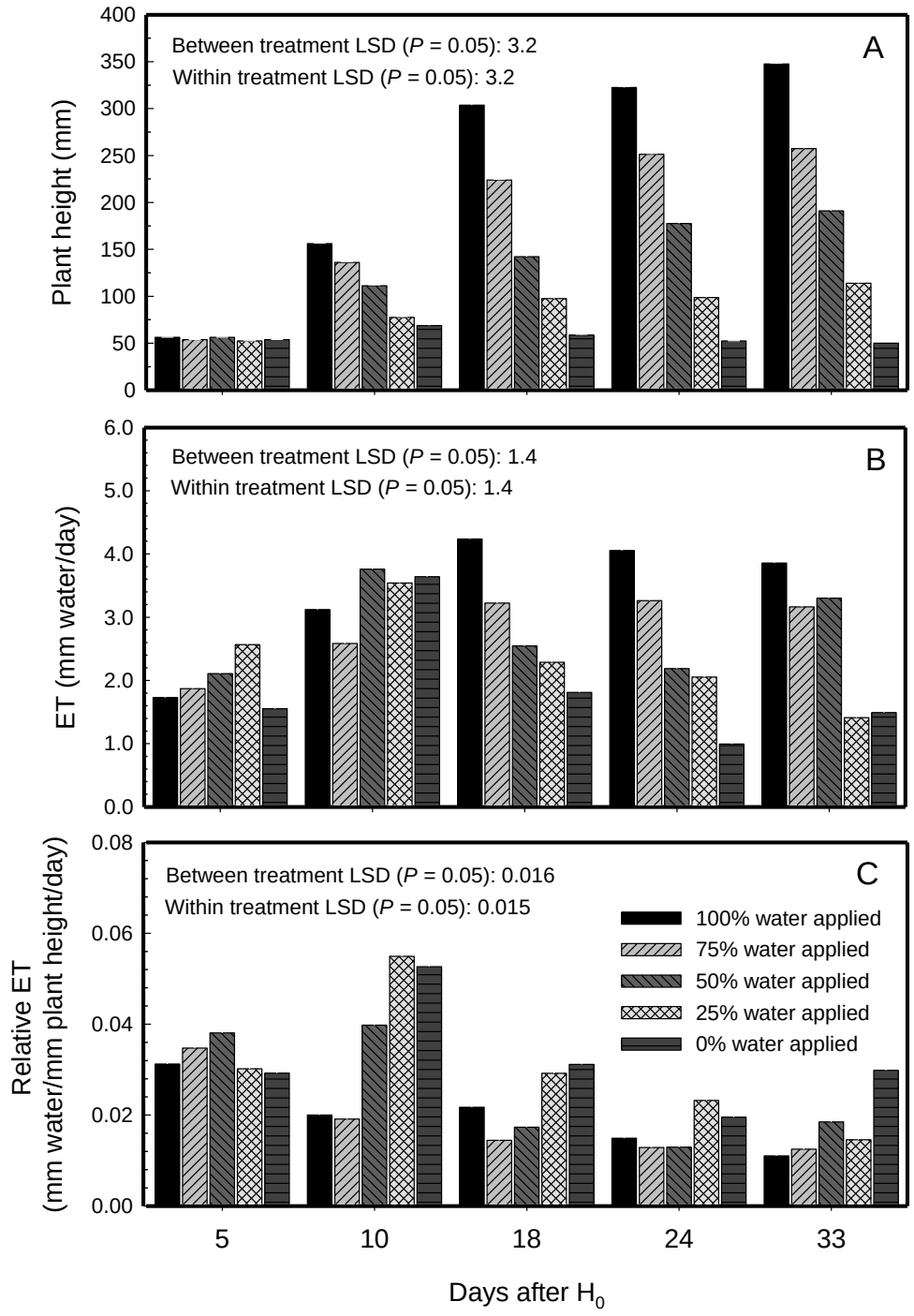

Figure 6.5. Plant height (A), estimated evapo-transpiration (ET; B) and relative ET (mm ET per mm of plant height; C) of lucerne (cultivars Grassland Kaituna and SARDI 10 averaged at each level of water deficit) regrowing under various levels of water deficit (100, 75, 50, 25 and 0% of the replacement water requirement applied) at 5, 10, 18, 24 and 33 days after H_0.

6.4. Discussion

Structural attributes of the canopy (biomass, yield components and shoot morphology) were all affected in proportion to the water deficits imposed, while photosynthesis parameters remained stable under all water deficits apart from the most severe deficit of no water applied. There was no interaction between cultivar and water deficit level for the canopy development or photosynthesis parameters measured. This indicates that for at least the two cultivars investigated, these processes are not susceptible to a genotype by water deficit interaction. The reduction in herbage DM accumulation with increasing levels of water deficit confirms previous research on the influence of water deficit on lucerne production in controlled environments (Cowett and Sprague 1962; Aranjuelo *et al.* 2006; Erice *et al.* 2007) and in the field (Carter and Sheaffer 1983a; Grimes *et al.* 1992; Brown *et al.* 2005a).

Both mass per shoot and number of shoots per plant were reduced by water deficit treatments. The reduction in both these components by the end of a regrowth cycle under water deficit has been previously reported (Perry and Larson 1974; Brown and Tanner 1983). However, in the current study, it appeared that shoot mass was unaffected by water deficit in the first seven days of regrowth. This suggests that factors external to the plant will have minimal influence during the early stages of shoot regrowth, provided that plants are healthy prior to defoliation.

Water applications of 50% and 25% of the replacement water requirement prevented the development of a full complement of shoots earlier in the regrowth cycle, compared with the 75% and 100% water application treatments. The shoots grown on the plants receiving 25% or 0% of the replacement water requirement were crown shoots with little contribution from axial shoots, while the plants receiving more water had a greater proportion of axial shoots. The timing of the development of a water deficit in the regrowth cycle also has an impact on shoot density. In the current experiment, water deficit imposed early in regrowth limited the number of shoots per plant. In the field, Brown and Tanner (1983) found that drought stress imposed early in the regrowth period reduced shoot density by 77%. However, it is important to note that when drought was imposed late in the regrowth cycle shoot

density was unaffected. In the current experiment, changes in the ratio of axial to crown shoots indicated that axial shoots developed in the later parts of the regrowth cycle. Deficit water applications of 25% or 0% of the replacement water requirement prevented axial shoots from developing on stressed plants, as growth of these plants had already ceased. The reduction in shoot density has consequences for herbage yield following the correction of water deficit. Plants with fewer shoots may have lower yield capacity following the correction of a water deficit. Avoiding a water deficit during early regrowth, thus allowing the plants to reach their full complement of shoots, would avoid this.

The slowdown in morphological development and increase in leaf-to-stem ratio observed in the current study is in line with previous research where water deficit was imposed early in the regrowth cycle (Carter and Sheaffer 1983a; Halim *et al.* 1989b). In the current experiment, both were due to a reduction in shoot elongation leading to shorter shoots. As the method to determine maturity of vegetative shoots is based on shoot length (Kalu and Fick 1981), water deficit led to a slowing in the rate of maturity. Those plants may have had a subsequent rapid increase in MSC and MSW as floral initiation began. However, the current experiment was not long enough to capture this developmental change using this relatively coarse developmental scale. The initiation of reproductive development on shoots would limit shoot regrowth following the correction of a water deficit, as any vegetative growth would have to come from the development of new shoots. This potentially explains the delay in recovery of growth following the correction of water deficit reported by some investigators (Metochis and Orphanos 1981; Halim *et al.* 1989b).

Leaf death only occurred in unwatered plants, resulting in a low leaf-to-stem ratio in that treatment. Drought-induced leaf death has been previously observed in lucerne (Brown and Tanner 1983; Halim *et al.* 1989a). However, in the current experiment it was only observed in unwatered plants, showing that lucerne can mediate drought stress through the rate of regrowth following defoliation, thereby protecting leaves from senescence.

Leaf senescence in plants that received no water severely reduced the CO_2 exchange rate. This was the only observed permanent effect of water deficit on photosynthetic parameters. Other authors (Aranjuelo *et al.* 2005; Erice *et al.* 2006) have reported that water deficit has no net impact on CO_2 exchange in regrowing lucerne receiving half its water requirement. In the current study, CO_2 exchange rates in the plants receiving 75% or 50% of the replacement water requirement were not significantly different to plants receiving 100% of the replacement water requirement, while the CO_2 exchange rate of the plants receiving 25% of the replacement water requirement were significantly reduced 19 days after H_0. This response however was transient, with similar rates of CO_2 exchange between the plants receiving 25% and 100% of the replacement water requirement observed at 24 days after H_0. Cultivar has minimal impact on CO_2 exchange rate under fully watered conditions (Ma *et al.* 2009). The absence of a cultivar effect on CO_2 exchange under water deficit conditions in the current experiment confirms that there are no genotype differences for CO_2 exchange rate in lucerne.

Maximum efficiency of photosystem II is a sensitive indicator of when and to what degree plants incur photosystem stress (Maxwell and Johnson 2000). Changes in Fv/Fm were only observed as leaf death occurred, indicating that unless the water deficit is severe enough to cause leaf death in regrowing lucerne, photosynthetic performance will not be reduced by a water deficit. Consistent with this finding, Aranjuelo *et al.* (2006) found that there was no influence on the Fv/Fm in lucerne after 10 days of under-watering by 50%.

It has been demonstrated that plants close stomata to reduce transpiration as leaf water potentials decrease in response to a water deficit (Ottman 1999), and this was observed in the current study with rates of stomatal conductance in the unstressed lucerne plants higher than in those exposed to a water deficit. Lazaridou and Noitsakis (2005) noted that stomatal conductance in lucerne is also reduced with increased maturity. However, this effect was not observed in the current study, with higher rates of stomatal conductance in the more mature plants. The maturity (as indicated by MSC and MSW) of the plants receiving their full requirement of water,

while statistically different, was only slightly greater than plants exposed to water deficit, with all still classed as vegetative after 35 days of regrowth.

While CO_2 exchange rate calculated per unit of leaf area and the efficiency of photosystem II was not affected by water deficit, in the long-term, overall plant photosynthesis would have been affected due to a reduction in leaf area associated with water deficit. This conclusion is the same as the one made by Erice *et al.* (2006), in that, reduced carbon assimilation associated with water deficit in lucerne is not due to a slowing in photochemistry, but simply to a reduction in leaf area. This conclusion can now be further expanded to say that there is no genotype effect on this response.

Lazaridou and Noitsakia (2005) observed that field-grown lucerne under drought conditions was able to maintain a favourable plant water potential while in the vegetative stages of morphological development. This is in agreement with the current study, where shoot water potentials of plants receiving at least some water remained similar to those of unstressed plants. This was achieved by plants mediating their canopy volume and leaf area to limit transpiration. While estimated ET was highest in the plants not under water deficit, when differences in canopy volume were accounted for, relative ET adjusted for height was similar across the levels of deficit, despite the limited stomatal conductance in the lucerne plants receiving a deficit water treatment. Grasslands Kaituna was able to exhibit greater control over shoot water potential in response to water deficit than SARDI 10. While this did not translate to an improvement in canopy structure or photosynthetic parameters in the current experiment, the ability to maintain more favourable shoot water potential under water limited conditions may have led to the greater yield of Grasslands Kaituna compared to SARDI 10 in the longer term field experiment described in Chapter 4.

The canopy forming processes investigated in this chapter where not affected by a genotype by water deficit interaction. Shoot water potential was the only parameter where genotype and water deficit interacted. Plant physiological processes other than those involved in canopy development must be responsible for

the genotype by environmental interactions observed the field experiments described in previous chapters. The following chapters will investigate taproot physiology and gene expression in regrowing lucerne exposed to a water deficit in an attempt to identify these processes.

CHAPTER 7

Partitioning of taproot reserves and crown bud development are affected by water deficit in regrowing lucerne

7.1. Introduction

In the cool temperate dairy regions of Australia, dryland grown lucerne is exposed to severe soil moisture deficits during summer. Water deficits reduce the number of shoots per plant and the mass per shoot in regrowing lucerne, and subsequently limit DM yield (Chapter 6). However, this response is not affected by genotype and as such an explanation of the superior performance of the more winter dormant cultivars under dryland conditions observed in Chapter 4 has not been identified.

Water deficit conditions slow the growth and development of shoots and reduce the number of shoots produced per plant (Lodge 1991; Gramshaw *et al.* 1993; Dhont *et al.* 2002; Chapter 6). Reduced plant growth consequently alters the demand and supply of taproot assimilates to shoots when compared to unstressed plants. Given the impact of drought on seasonal changes in taproot assimilates highlighted in Chapter 5 and the genotype effect on taproot reserve pools (Cunningham *et al.* 1998), the cyclic pattern of defoliation-induced assimilate depletion and re-accumulation in lucerne exposed to water deficits may be mediated by genotype.

Crown buds are also an important plant organ in lucerne regrowth with the majority of shoots arising from crown buds when best management practices for defoliation are employed (Chapter 4) and when plants are exposed to a water deficit (Chapter 6). Genotypic differences for the development of crown buds

during periods of water deficit may affect canopy forming processes in subsequent regrowth cycles.

This chapter will examine the hypothesis that accumulation of taproot reserves and rate of crown bud development will increase with exposure to a water deficit during a regrowth cycle. In addition, it will further examine the hypothesis that the magnitude of these responses will be proportional to the magnitude of the imposed water deficit. The objectives of this chapter were to investigate the utilisation and re-accumulation of taproot reserve pools and the development of crown buds of two contrasting lucerne cultivars regrowing with a range of water deficits following defoliation.

7.2. Materials and methods

7.2.1. Experimental design and management

The experimental design, timeline, management of experimental plants and application of water deficit treatments are described in Chapter 6.

7.2.2. Measurements

At each destructive sampling, after the removal of shoots (described in Chapter 6) the roots and crowns were washed free of soil. Taproots were separated from the crown just below the lowest crown bud or shoot branch. At harvests occurring at H_0, 14 days after H_0 and 35 days after H_0 for plants of both cultivars receiving 100% or 25% of the replacement water requirement two randomly selected taproots were set aside (see Chapter 8). For all other treatments and destructive harvests all taproots were collected. Taproots were frozen on dry-ice and stored at -80°C until freeze-drying. Any buds present on the crowns were removed and grouped into green or white buds, counted, frozen on dry ice and stored at -80^{o}C until freeze-drying. Buds were defined as an outgrowth originating on the crown without visible leaves. Crowns were dried at 60°C for 48 hours in a forced-air oven. Freeze dried taproots were mechanically ground through a 1 mm screen.

Taproot ethanol soluble sugars were extracted and taproot sugar and starch concentration determined following the methods described in Chapter 5. Total non-structural carbohydrates were considered to be the sum of soluble sugars and starch. Soluble sugar, starch and TNC concentrations are expressed on a dry weight basis. Soluble proteins and amino acids were extracted and concentration determined following the methods described in Chapter 5. In addition to the taproot reserve concentrations, reserve contents were calculated as concentration × taproot DM.

For SDS-PAGE analysis (Plate 7.1), three replications of 20 μg of extracted protein from ground root tissue sampled 35 days after H_0 as well as a control sample from plants harvested prior to H_0 mixed one-to-one with SDS-PAGE sample loading buffer were separated in gels as described in Chapter 5. Gels were scanned and optical intensity of bands corresponding to the high, middle and low molecular weight VSPs was determined by following the exact procedures described in Chapter 5.

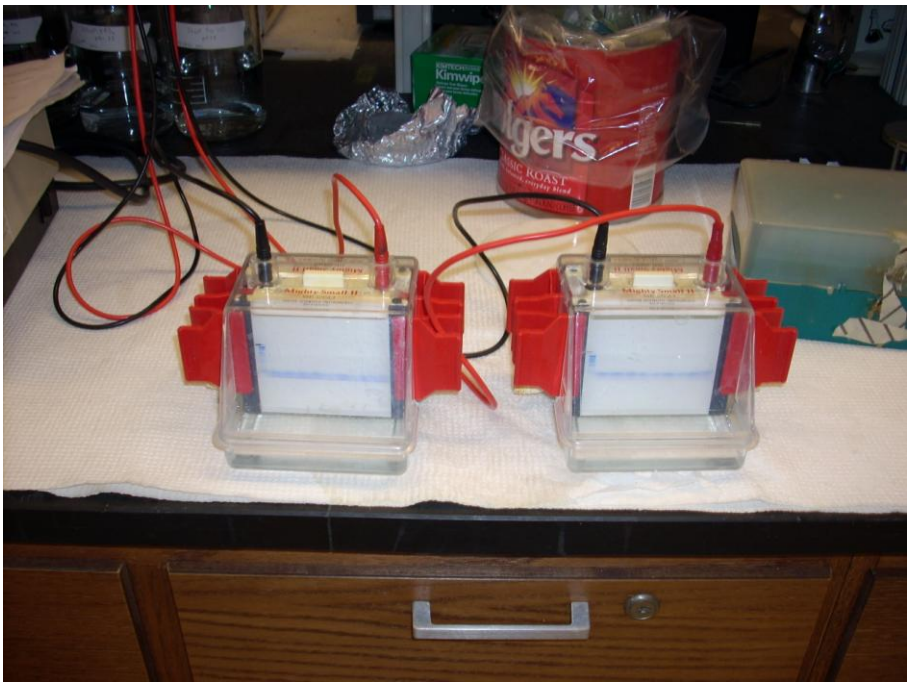

Plate 7.1. Part way through the separation of proteins by SDS-PAGE for the analysis of lucerne taproot soluble protein pool composition.

7.2.3. Statistical analysis

All taproot, crown and bud response variables were analysed using ANOVA of a randomised complete block design with level of water deficit, cultivar and harvest randomised within each block. Green bud mass was subjected to a square root transformation before analysis. To enable comparisons between gels and account for gel-to-gel variation, optical intensity values from the high, middle and low molecular weight VSP from each sample were compared to the H_0 sample on that gel and the percentage difference calculated. Analysis of variance of a randomised complete block design was then performed on the percentage difference, with cultivar and water deficit as factors. Fisher's LSD was used to identify significant differences between treatment means when ANOVA indicated a significant effect. Unless otherwise stated, significant effects were accepted when $P < 0.05$. Data analyses were achieved using Genstat 9.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK).

7.3. Results

7.3.1. Root and crown dry matter

There was a significant ($P < 0.001$) water deficit by harvest interaction effect on taproot and crown DM (Table 7.1). Taproot DM increased through the regrowth period in plants receiving 25% or greater of the replacement water requirement, while taproot growth did not occur in plants that received no water (Table 7.2). By 35 days after H_0 there was a significant difference in taproot mass per plant between plants receiving 100% of the replacement water requirement and all other levels of water deficit. At this time taproot mass of plants receiving 75% or 50% of the replacement water requirement were greater than those plants receiving less water.

Crown mass per plant (Table 7.2) increased in the first 21 days after H_0 for all the levels of water deficit as new crown shoots developed following

defoliation. Further increases in crown mass of plants receiving 100%, 75% and 50% of the replacement water requirement occurred during the remaining regrowth period. At 28 days after H_0, crown mass of plants receiving at least 50% or more of the replacement water requirement had significantly increased 1.6- to 2.1-fold times that of plants at H_0. There was no significant interaction between cultivar and water deficit at each assessment (Table 7.1).

Table 7.1. Summary of ANOVA of crown and taproot results monitored five times over 35 days from H_0 for two lucerne cultivars regrowing with five levels of water deficit.

		Taproot											Crown				
Source of		DM[‡]	TNC[†]		Starch		Sugar		Protein		Amino acids		DM	Green bud		White bud	
variation	df		Conc.	Total	Conc.	Total	Conc.	Total	Conc.	Total	Conc.	Total		No.	DM	No.	DM
Cultivar (C)	1	**	ns	*	ns	*	ns	**	**	***	***	ns	ns	ns	ns	**	ns
Water deficit (W)	4	***	**	***	***	***	***	***	*	***	***	***	***	***	***	**	ns
Harvest (H)	5	***	***	***	***	***	***	***	***	***	***	***	***	***	***	**	*
C x W	4	ns[§]	ns	ns	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
C x H	5	ns	*	ns	*	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
W x H	20	***	***	ns	***	**	***	***	***	ns	***	ns	***	***	***	***	***
C x W x H	20	ns	**	ns	**	ns	***	ns	**	ns	**	ns	ns	ns	ns	ns	ns

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the 0.001 probability level.
TNC[†], total non-structural carbohydrate.
DM[‡], dry matter
ns[§], no significant effect.

7.3.2. Taproot carbohydrate concentration and availability

There was a significant water deficit by harvest date interaction effect on taproot TNC ($P < 0.001$), soluble sugar ($P < 0.001$) and starch ($P < 0.001$) concentrations (Table 7.1). Averaged over all levels of water deficit the concentration of taproot TNC decreased in SARDI 10 after defoliation before increasing above pre-defoliation levels by 21 days after H_0 (Table 7.3). Grasslands Kaituna had no decrease in TNC concentration post-defoliation and increased above the pre-defoliation concentration by 21 days after H_0 when data was averaged over all levels of water deficit.

Table 7.2. Taproot and crown dry mass per plant of lucerne (two cultivars averaged at each level of water deficit) regrowing with various levels of water deficit; 100, 75, 50, 25 and 0% of the replacement water requirement applied.

Treatment	Days after H_0†					
	Pre-defoliation	7	14	21	28	35
	Taproot mass (g)					
100% water	0.41	0.35	0.33	0.54	0.67	1.20
75% water		0.37	0.37	0.63	0.90	0.94
50% water		0.49	0.42	0.57	0.79	0.84
25% water		0.47	0.44	0.53	0.62	0.67
0% water		0.42	0.46	0.43	0.49	0.48
LSD (P=0.05)				*0.15*		
Grasslands Kaituna	0.42	0.41	0.47	0.57	0.73	0.85
SARDI 10	0.39	0.43	0.34	0.51	0.66	0.80
LSD (P = 0.05)				ns‡		
	Crown mass (g)					
100% water	0.17	0.14	0.20	0.26	0.28	0.37
75% water		0.15	0.18	0.25	0.34	0.39
50% water		0.16	0.22	0.27	0.36	0.32
25% water		0.15	0.20	0.22	0.25	0.26
0% water		0.15	0.22	0.27	0.24	0.31
LSD (P=0.05)				*0.05*		
Grasslands Kaituna	0.18	0.14	0.22	0.27	0.29	0.33
SARDI 10	0.16	0.16	0.19	0.23	0.30	0.33
LSD (P = 0.05)				ns		

H_0†, Second defoliation, 108 DAE
ns‡, no significant effect.

Table 7.3. Total non-structural carbohydrate (TNC) concentration (dry mass basis) in the taproots of lucerne (two cultivars averaged at each level of water deficit) regrowing with various levels of water deficit; 100, 75, 50, 25 and 0% of the replacement water requirement applied.

Treatment	Days after H_0†					
	Pre-defoliation	7	14	21	28	35
	Taproot TNC (mg/g)					
100% water	297	276	277	363	382	373
75% water		239	279	331	422	423
50% water		267	275	333	413	389
25% water		308	280	317	329	356
0% water		280	258	309	346	335
LSD (P=0.05)				44		
Grasslands Kaituna	288	271	292	320	377	388
SARDI 10	307	277	256	341	380	363
LSD (P = 0.05)				28		

H_0†, Second defoliation, 108 DAE

The increase in TNC concentration following defoliation was less in the plants receiving 25 or 0% of the replacement water requirement compared to those that received 75% of the replacement water requirement (Table 7.3). Between 14 and 28 days after H_0 the concentration of TNC increased in the taproot. The water deficit treatment influenced both the time to replenishment of TNC and the level of replenishment. Plants receiving 25% and 0% of the replacement water requirement had a smaller increase in TNC above pre-defoliation levels and this increase occurred later in the regrowth period compared to the other watering treatments. Averaged across both cultivars, plants receiving 100, 75 or 50% of the replacement water requirement had a greater TNC concentration than the plants that received 25% of the replacement water requirement by 28 days after H_0 and the plants that received 0% of the replacement water requirement at 35 days after H_0. Despite this, taproot TNC concentration for all levels of water deficit returned to pre-defoliation concentrations by 14 days after H_0.

Taproot soluble sugar concentrations of Grasslands Kaituna plants that were receiving 25 and 0% of the replacement water requirement were greater than those of

plants receiving 100% water at 14 and 21 days after H_0 (Figure 7.1). For SARDI 10 this increase was only observed in plants that received no water. By 28 days after H_0 the increase in taproot soluble sugars in the Grasslands Kaituna plants receiving 25% of the replacement water requirement had subsided, with similar concentrations evident between all plants that received at least some water. From 21 days after H_0 starch concentration was greater in the taproot of plants that received at least some water, apart from plants of SARDI 10 at 28 days after H_0 that received 25% of the replacement water requirement (Figure 7.2).

Averaged across all watering treatments and both cultivars, taproot TNC content increased 21 days after H_0 (data not shown). Averaged over the 35 days of regrowth Grassland Kaituna had consistently more taproot TNC (189 to 168 mg/plant), soluble sugars (44.8 to 39.7 mg/plant) and starch (144 to 128 mg/plant) when compared to SARDI 10. Water deficit and harvest interacted to affect ($P < 0.01$) soluble sugar and starch content (Table 7.1). The total amount of soluble sugars in taproots increased between 7 and 14 days after H_0 in all treatments apart from plants that received 100% of the replacement water requirement. The plants that received no water also had a 2.6-fold increase in soluble sugar content between H_0 and 28 days after H_0 (Table 7.4). Plants receiving 25% of the replacement water requirement maintained a constant taproot starch content through the regrowth period, while plants receiving 50% or more of the replacement water requirement had increased taproot starch content 28 days after H_0 (Table 7.4).

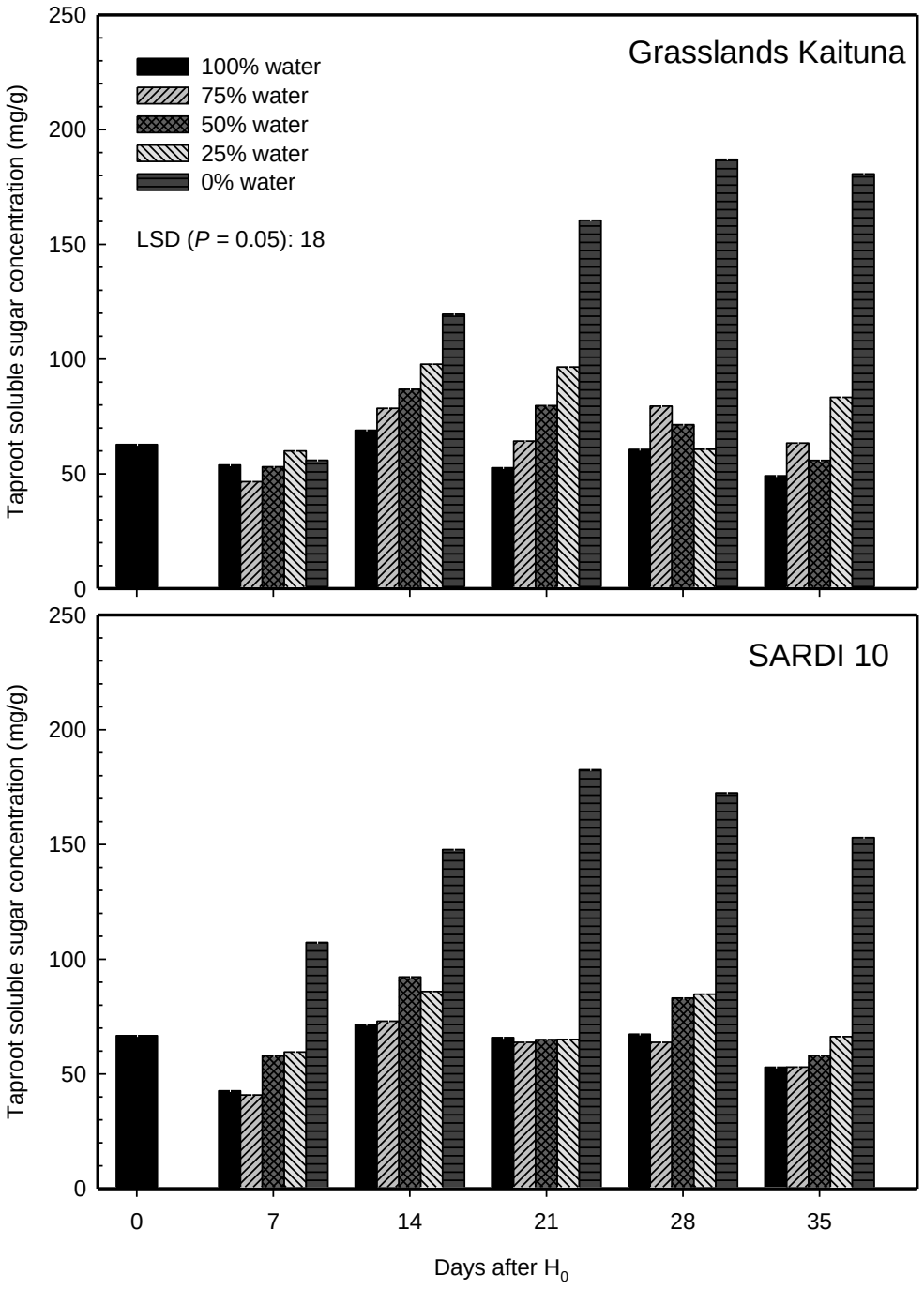

Figure 7.1. Concentration of ethanol-soluble sugars in the taproots of Grasslands Kaituna and SARDI 10 lucerne regrowing at various levels of water deficit; 100, 75, 50, 25 and 0% of their replacement water requirement applied. The first thick solid bar represents the plants prior to H_0 (108 DAE).

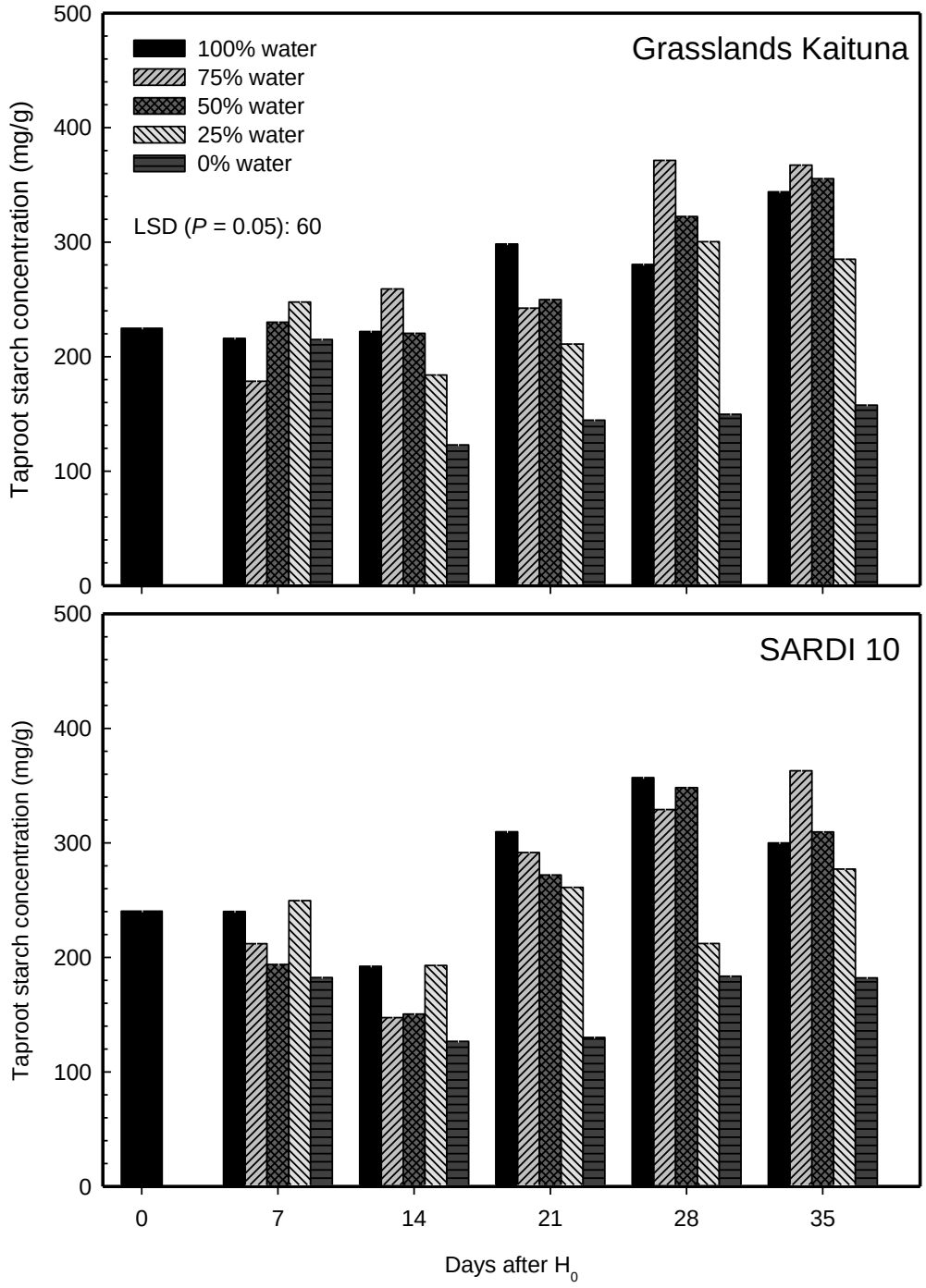

Figure 7.2. Concentration of starch in the taproots of Grasslands Kaituna and SARDI 10 lucerne regrowing at various levels of water deficit; 100, 75, 50, 25 and 0% of their replacement water requirement applied. The first thick solid bar represents the plants prior to H_0 (108 DAE).

Table 7.4. Total amount of taproot sugar and starch (mg/plant) in lucerne (two cultivars averaged at each level of water deficit) regrowing with various levels of water deficit; 100, 75, 50, 25 and 0% of the replacement water requirement applied.

Treatment	Days after H_0[†]					
	Pre-defoliation	7	14	21	28	35
	Total taproot sugar (mg/plant)					
100% water	33.1	26.5	40.2	38.6	36.9	34.0
75% water		27.7	46.0	37.0	47.2	35.0
50% water		28.1	53.6	41.1	49.3	34.4
25% water		31.7	47.9	44.1	35.3	41.2
0% water		34.6	60.4	69.7	87.5	73.3
LSD (P = 0.05)				*14.1*		
Grasslands Kaituna	33.0	29.0	54.3	48.2	55.1	49.2
SARDI 10	33.2	30.5	45.0	44.0	47.4	38.0
LSD (P = 0.05)				ns[‡]		
	Total taproot starch (mg/plant)					
100% water	117	116	130	199	193	209
75% water		121	129	151	238	230
50% water		109	118	152	215	199
25% water		138	98	136	127	150
0% water		82	56	56	79	77
LSD (P = 0.05)				*57*		
Grasslands Kaituna	116	115	129	135	175	194
SARDI 10	117	111	83	141	165	151
LSD (P = 0.05)				ns		

H_0[†]Second defoliation, 108 DAE
ns[‡], no significant effect

7.3.3. Taproot soluble protein and amino acid concentration and availability

Protein concentration was affected ($P < 0.001$) by a water deficit by harvest interaction (Table 7.1). Taproot soluble protein concentration averaged across cultivars was initially reduced after defoliation and then subsequently returned to initial pre-defoliation levels by 21 days after H_0 for all treatments (Figure 7.3). Plants receiving 75% or 50% of the replacement water requirement also had a further reduction in protein concentration between 7 and 14 days after H_0. For plants that received no water, taproot soluble protein concentration decreased again between 28

and 35 days after H_0 to a concentration less than that at H_0. A similar pattern was observed in the total amount of taproot soluble protein per plant (data not shown).

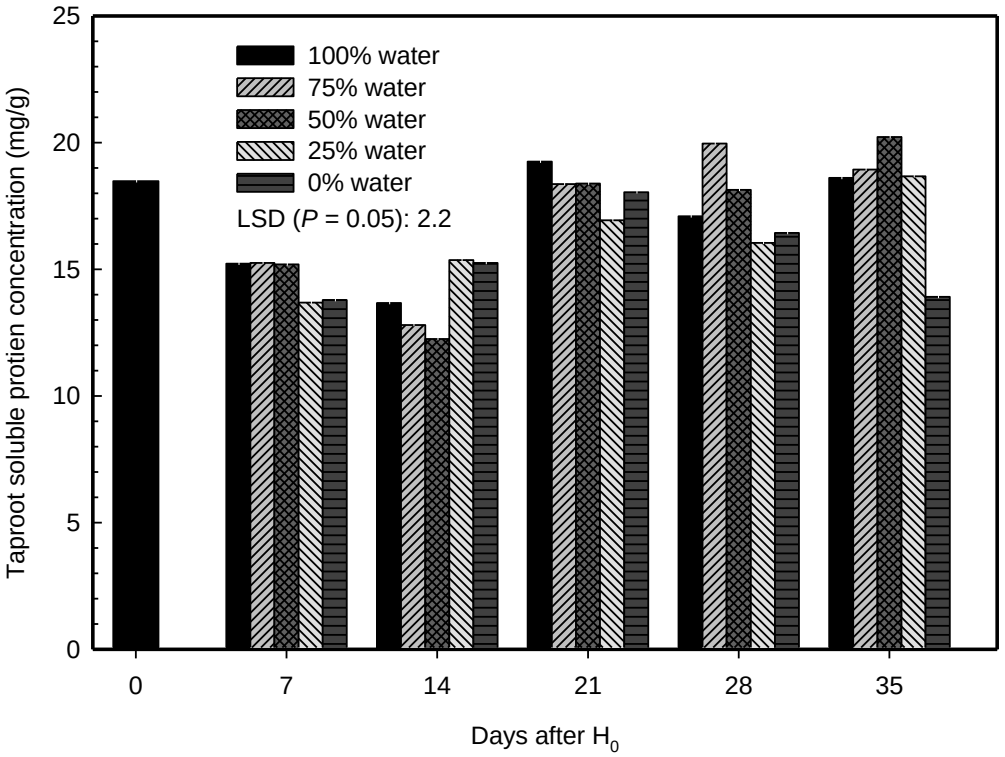

Figure 7.3. Concentration of soluble protein in taproots of lucerne plants (cultivars averaged in each level at water deficit) regrowing at various levels of water deficit; 100, 75, 50, 25 and 0% of their replacement water requirement applied. The first thick solid bar represents the plants prior to H_0 (108 DAE).

Water deficit altered the composition of the soluble protein pool by 35 days after H_0 in both cultivars (Figure 7.4). Between H_0 and 35 days after H_0 the percentage increase in optical intensity of the bands corresponding to the middle and low molecular weight VSP was significantly ($P < 0.05$) greater for plants receiving 50 and 25% of the replacement water requirement compared to the plants that received 100% of the replacement water requirement (Table 7.5).

There was a three-way water deficit by cultivar by harvest interaction affecting amino acid concentration ($P < 0.01$; Table 7.1). Despite the interaction, by 35 days after H_0 for both cultivars amino acid concentration was only significantly different between the treatments that received no water compared to the other water deficit treatments (Figure 7.5). The total amount of amino acid in the taproots of both cultivars significantly increased from H_0 to 35 days after H_0 in all levels of water deficit apart from the plants that received no water (data not shown). In these plants by 35 days after H_0, taproot amino acid content averaged across both cultivars decreased by more than 30% (to 0.066 mmol/plant) compared to all other treatments (average of 0.107 mmol/plant).

Table 7.5. Percentage difference between the optical intensity of the bands from SDS-PAGE analysis corresponding to high, middle and low molecular weight vegetative storage proteins in the taproots of lucerne plants harvest just before H_0 (108 DAE) compared to plants receiving 100, 75, 50, 25 and 0% of replacement water for 35 days after H_0 , Optical intensities are averaged over two cultivars.

Treatment	Vegetative storage protein		
	High	Middle	Low
	Arbitrary units of optical intensity		
H_0	1829	1566	1129
	Optical intensity, % above H_0		
100% water	6	24	29
75% water	21	47	58
50% water	55	97	99
25% water	47	89	84
0% water	63	55	24
LSD ($P = 0.05$)	ns[†]	48	45

ns[†], no significant effect.

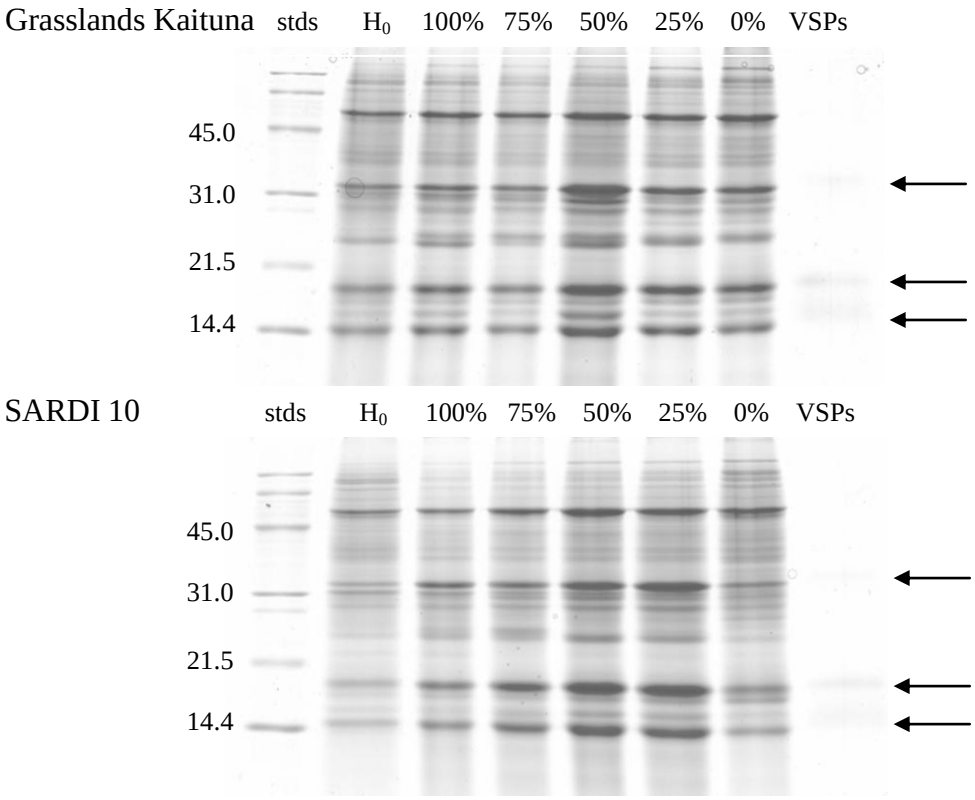

Figure 7.4. SDS-PAGE analysis of buffer soluble proteins extracted from ground taproot tissue of both lucerne cultivars from 35 days after H_0 at various levels of water deficit compared to before H_0. Samples were loaded from left to right as follows: molecular weight standards (stds), 100% of the replacement water requirement prior treatments (H_0), 100%, 75%, 50%, 25% and 0% of the replacement water requirement applied for 35 days after H_0 and purified lucerne vegetative storage proteins (VSPs). Numerals on the left represent the size of molecular weight standards (kD) and the arrows on the right highlight the 15, 19 and 32 kD lucerne VSPs.

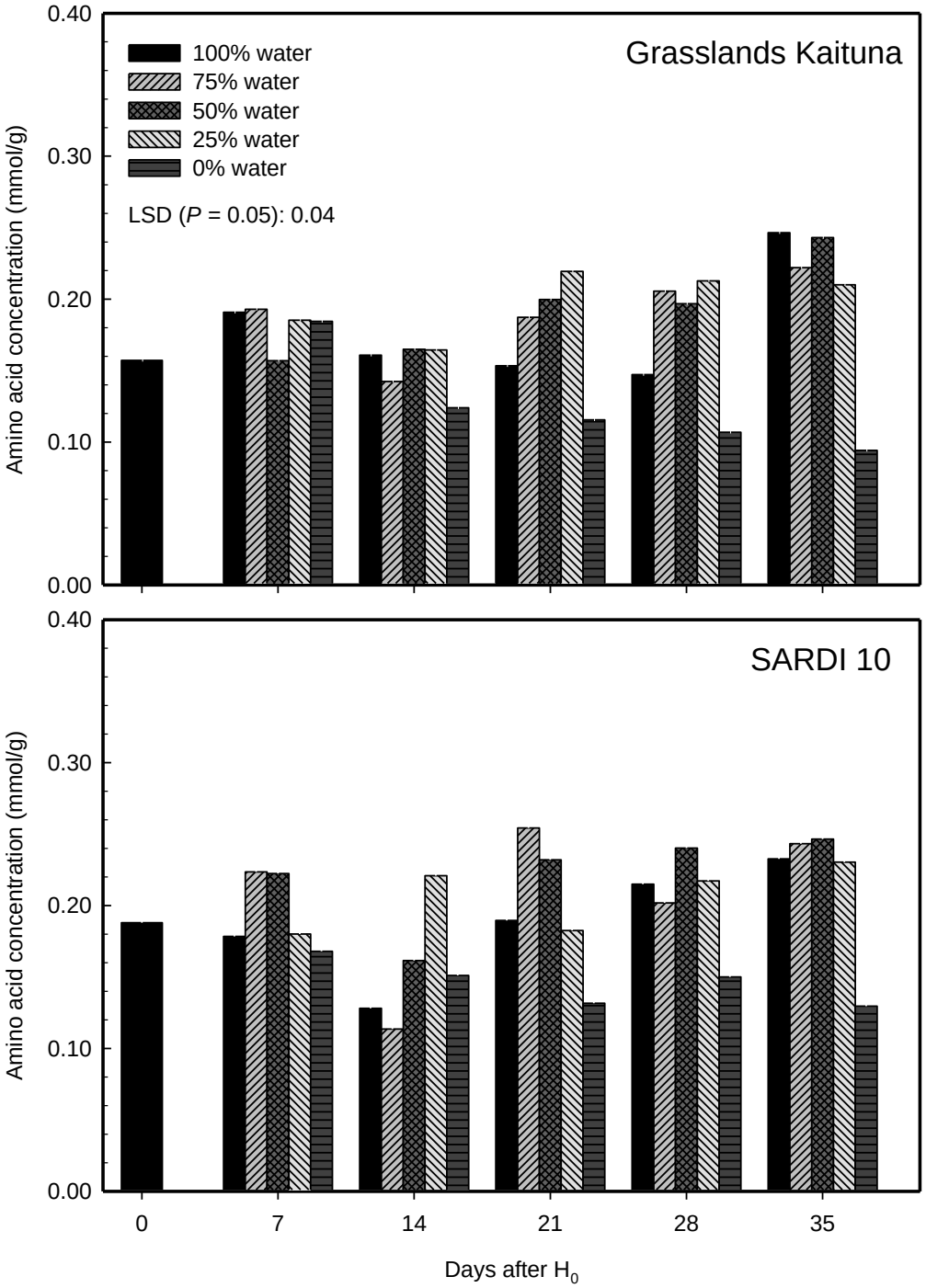

Figure 7.5. Concentration of amino acid in the taproot of Grasslands Kaituna and SARDI 10 lucerne regrowing at various levels of water deficit; 100, 75, 50, 25 and 0% of their replacement water requirement applied. The first thick solid bar represents the plants prior to H_0 (108 DAE).

7.3.4. Crown bud development

The development of green crown buds (both DM and number) was affected by a two-way interaction between water deficit and harvest date ($P < 0.001$; Table 7.1). Plants receiving no water had no green buds present on the crowns by 21 days after H_0. Plants from the other water deficit treatments maintained similar numbers of green buds to that of the unstressed plants until 28 days after H_0 (Table 7.6). At 35 days after H_0 plants receiving 75% of the replacement water requirement had greater numbers of buds than the other water deficit treatments but were similar to the plants receiving 100% of the replacement water requirement. The numbers of crown buds on plants receiving 100% of the replacement water requirement decreased between 28 and 35 days after H_0. There was no cultivar effect on the number of green crown buds per plant.

The mass of individual crown buds after recovery from defoliation increased in all treatments that received water (Table 7.6). Buds on the plants receiving no water decreased in mass before becoming undetectable. As with the number of green buds per plant, there was no detectable difference in the mass per green bud between the two cultivars.

White buds (data not shown) were rarely observed in this experiment (averaging 0.11 buds/plant) and when found were small (averaging 0.15 mg/bud). Numerically, the fewest white buds were on plants receiving no water and the cultivar Grasslands Kaituna had more white buds than SARDI 10.

Table 7.6. The number and dry mass of green crown buds present on lucerne (cultivars averaged at each level of water deficit) regrowing with different levels of water deficit; 100, 75, 50, 25 and 0% of the replacement water requirement applied. Values in parentheses are the means after transformation using square root + 0.5.

	Days after H_0[†]					
Treatment	Pre defoliation	7	14	21	28	35
	Green buds per plant					
100% water	0.48	0.29	0.25	0.33	1.09	0.74
75% water		0.32	0.29	0.22	0.63	0.95
50% water		0.25	0.40	0.17	0.70	0.40
25% water		0.23	0.33	0.27	0.76	0.49
0% water		0.19	0.10	0.00	0.10	0.00
LSD (P = 0.05)				*0.27*		
Grasslands Kaituna	0.47	0.42	0.36	0.51	0.43	0.36
SARDI 10	0.50	0.38	0.35	0.36	0.39	0.38
LSD (P = 0.05)				ns[‡]		
	Green bud mass (mg/bud)					
100% water	0.40	0.11 (0.78)	0.22 (0.85)	0.29 (0.89)	0.83 (1.15)	0.82 (1.15)
75% water		0.29 (0.89)	0.22 (0.85)	0.42 (0.96)	0.68 (1.08)	0.68 (1.09)
50% water		0.21 (0.84)	0.23 (0.86)	0.24 (0.86)	0.65 (1.07)	0.50 (1.00)
25% water		0.20 (0.83)	0.30 (0.90)	0.28 (0.89)	0.94 (1.20)	0.72 (1.10)
0% water		0.24 (0.86)	0.11 (0.78)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)
[§]LSD (P=0.05)				*0.15*		
Grasslands Kaituna	0.28	0.22 (0.85)	0.24 (0.86)	0.25 (0.87)	0.59 (1.04)	0.51 (1.00)
SARDI 10	0.51	0.20 (0.84)	0.19 (0.83)	0.23 (0.85)	0.58 (1.04)	0.53 (1.02)
[§]LSD (P=0.05)				ns		

H_0[†]Second defoliation, 108 DAE.
ns[‡], no significant effect.
[§]LSD is for the transformed data within the parenthesis.

7.4. Discussion

While water deficit affected the partitioning of reserve pools, this effect was not proportional to the water deficits imposed. The only major difference occurred between those plants receiving no water and those receiving 100% of the replacement water requirement. Only minor differences were observed between the other water deficit treatments. In addition, cultivar had only a minimal influence on the response of the reserve pools to water deficit. The pattern of depletion and reaccumulation of taproot N pools after defoliation and during subsequent herbage regrowth was similar to previously published findings for the influence of defoliation on taproot reserves (Hendershot and Volenec 1993). In the current experiment water deficit affected the pattern in which N pools were mobilised from the taproots during regrowth. While all treatments had a reduction in protein concentration within the first seven days after defoliation a further reduction in protein concentration was observed between seven and 14 days after defoliation for plants receiving 75% and 50% of the replacement water requirement. This was most likely a consequence of altered demand for taproot reserves to support shoot growth. With severe moisture stress, the growth rate of lucerne shoots is slowed, allowing the plant to maintain an adequate plant water balance (Chapter 6), hence the N requirement to support the production of new shoot tissue was reduced, while plants that received 100% of the replacement water requirement were able to quickly recover from defoliation.

As water deficit increased, the proportion of low and middle molecular weight VSPs in the taproot soluble protein pool increased compared to unstressed plants, as long as plants received some water. The preferential accumulation of two out of three VSPs was unexpected. Previous studies have shown that all three VSPs responded in the same manner to environmental stimuli (Kalengamaliro *et al.* 1997; Cunningham *et al.* 1998; Haagenson *et al.* 2003a). The method of optical analysis of these gels may not have been sensitive enough to capture an increase in the intensity of the high molecular weight VSP band from water stressed plants as flat bed scanners using white light are not as sensitive as commercially available densitometry scanners (Vincent *et al.* 1997; Tan *et al.* 2007).

The increase in relative abundance of VSPs with water deficit is consistent with the findings described in Chapter 5 and those of Erice *et al.* (2007). Erice *et al.* (2007) suggested that the increase in VSPs indicates either a contribution of these proteins to the plant's tolerance mechanisms of drought stress, or that they are a method of salvaging and recycling N resources from stressed tissue. The observations of a regrowing lucerne canopy (Chapter 6) suggests that the shoots were not stressed despite the water deficit, as the plants slowed growth rate to maintain a favourable shoot water potential. This stress avoidance would reduce the need for N redistribution. As such an alternative hypothesis is that the increase in VSPs is one key consequence of reduced canopy growth of defoliated lucerne challenged by water deficits.

Erice *et al.* (2007) reported that taproot soluble sugar concentrations during regrowth were unaffected by a water deficit. However, in the current study, after 35 days of regrowth taproot sugar concentration increased in plants receiving 25% of the replacement water requirement. The discrepancy between these two findings could be explained by the differing water deficit treatments. Erice *et al.* (2007) only investigated a single water deficit level, 50% of required water, whereas the response in the current experiment was only observed in plants receiving 25% or less of the replacement water requirement. Soluble sugars (primarily sucrose) have a role in the maintenance of osmotic potential and cell turgor in plants with a water deficit (Scott 2000). In the current experiment, the increase in soluble sugars was minor in plants that received at least some water. Plants that had no water applied however, had a clear increase in soluble sugar despite a reduction in photosynthesis and the ultimate death of above ground growth as reported in Chapter 6. This response may have been a cell turgor maintenance response in the taproots of these plants exposed to severe water deficit by the degradation of taproot starch and the salvaging of carbohydrate from the dying shoots.

The response of taproot starch concentration to water deficit differed between cultivars. SARDI 10 plants that received 75, 50 and 0% of the replacement water requirement exhibited a starch depletion pattern between seven and 14 days of regrowth, which is consistent with previously published reports on starch depletion

following defoliation (Boyce and Volenec 1992; Yamamoto *et al.* 1996). Grasslands Kaituna, however, exhibited no significant decline in taproot starch concentration as long as plants received some water. Accumulation of starch in the taproots of lucerne during the summer is associated with winter survival in the northern hemisphere (Boyce and Volenec 1992), while the amount of starch depleted during regrowth is negatively correlated to persistence in drought-prone environments (Boschma and Williams 2008). Grasslands Kaituna (a semi winter-dormant cultivar) may be a more persistent cultivar than SARDI 10 (a highly winter-active cultivar) when exposed to longer-term drought conditions due to the maintenance of high concentrations of starch. The results presented here and those of Boschma and Williams (2008) may provide a basis for the recommendation that in subtropical Australia, winter-dormant genotypes are best suited to extensive pastoral enterprises and winter-active genotypes are best adapted to irrigated hay production systems (Lloyd *et al.* 2002) and supports the similar conclusions made in Chapters 3 and 4 for cool temperate environments.

In this experiment taproot TNC concentration was not increased by water deficit at the end of the regrowth cycle, and while this was also observed by Erice *et al.* (2007), both studies are in contrast with previous field studies (Cohen *et al.* 1972) that observed an increase in TNC with water deficit. This discrepancy may be explained by the duration of the experiments. Cohen *et al.* (1972) monitored field grown lucerne over a 90 day period while the current experiment and that of Erice *et al.* (2007) had a duration of 35 and 30 days, respectively. As taproot reserves are depleted following defoliation then re-accumulated during regrowth, the accumulation of carbohydrates in the taproots of plants subjected to a water deficit would require several regrowth cycles to develop, as was the case in the experiment described by Cohen *et al.* (1972), but not in the current experiment or that described by Erice *at al.* (2007).

The role of taproot carbohydrates in the regrowth of lucerne is to support the respiration of the perennial tissues (Avice *et al.* 1996a). As such, plants with a greater concentration of taproot carbohydrate will be able to better support each individual cell that makes up the perennial organs. An increase in taproot TNC

concentration by the end of the regrowth period was only observed in the plants that received 75% of the replacement water requirement after 35 days of regrowth. The other water deficit treatments either had no increase, or had a decrease in taproot TNC concentration after 35 days of regrowth, and as such these plants are likely to have a slower recovery after the correction of a water deficit.

Even minor water deficit stress like those observed in plants that received 75% of the replacement water requirement inhibited the development of new green buds on the crowns. As the water deficit stress increased, the delay in development of green crown buds also increased and this was in proportion to the level of stress imposed. For the plants that received their full water requirement, green bud numbers decreased at the end of the regrowth cycle. This decrease was due to the already present population of crown buds elongating into new shoots. Bud size, unlike bud number, appeared unaffected by water deficit as long as the plants received at least some water. Genotype does not appear to influence bud numbers or size in autumn or winter (Juan *et al.* 1994; Cunningham *et al.* 1998). From the current experiment it would appear that this is also true for these two cultivars growing in summer conditions and exposed to a water deficit following defoliation. The results from this experiment and others (Juan *et al.* 1994; Cunningham *et al.* 1998) shows that winter dormancy genotype does not interact with environmental conditions to influence crown bud development.

The current experiment has identified that water deficit only influenced the partitioning of taproot reserves under the most severe water deficits (i.e. no water applied during regrowth). Plants that received at least some water lacked or had only a minor effect from water deficit on how taproot reserves were depleted and reaccumulated. However, a moderate water deficit altered the composition of the soluble protein pool after 35 days of regrowth, with an increase in the relative abundance of VSPs. Crown bud development was slowed when plants received 50% or less of the replacement water requirement. Through the current experiment and the results presented in Chapter 6 it is apparent that genotype has minimal interaction with water deficit to affect the process of canopy formation and carbohydrate and N reserve utilisation an accumulation. The short time frame of this glasshouse study

may have prevented the observations of an interaction at the physiological level. A molecular research approach may provide unique insights into possible genotype by water deficit interactions. The next chapter will examine the adaptation of several key physiological processes within the two cultivars examined at a gene expression and translational level.

CHAPTER 8

Abundance and transcription of key proteins and genes during regrowth of lucerne when exposed to a water deficit

8.1. Introduction

Soil moisture deficits during regrowth of lucerne (*Medicago sativa* L.) reduce shoot growth and yield (Brown and Tanner 1983; Carter and Sheaffer 1983b; Durand *et al.* 1989; Chapters 4 and 6), but have minimal effect on taproot carbohydrate and N reserves, unless the water deficit is severe (50% to 25% of the plant's full water requirement; Erice *et al.* 2007; Chapter 7). In lucerne, taproot soluble proteins are the most abundant and important N reserve pool (Hendershot and Volenec 1993). While the size of the taproot soluble protein is unaffected by water deficits, the polypeptide composition of this reserve pool is (Chapter 7).

The composition of soluble proteins in perennial organs of many plant species is affected by drought, resulting in preferential accumulation of specific polypeptides, including dehydrins, that maintain membrane stability during cell dehydration (Close 1996; 1997; Nylander *et al.* 2001; Volaire 2002; Norton *et al.* 2006). In lucerne, an accumulation of dehydrins during drought stress has yet to be verified. However, accumulation of VSPs during drought stress has been observed in controlled environments (Erice *et al.* 2007; Chapter 7) and in field-grown lucerne (Chapter 5). While the cellular purpose for the increased VSPs in lucerne during drought has not been clearly identified, suggestions include a direct role in cell adaption to dehydration (Erice *et al.* 2007), and/or as a mechanism for recycling N from stressed tissues (Erice *et al.* 2007; Chapter 7). For either one of these propositions to be correct, genes encoding for VSPs should be up-regulated in plants exposed to drought when compared to plants grown under fully watered conditions.

The process of cold acclimation and associated winter dormancy increases the concentration of VSPs in lucerne taproots (Hendershot and Volenec 1992), as well as increasing the transcript abundance of genes belonging to the cold regulated

(COR), cold acclimation-specific (CAS) and cold acclimation-responsive (CAR) gene families (Castonguay *et al.* 2006). The punitive proteins predicted from their gene sequences possess homology to dehydrins, nuclear signalling proteins and enzymes involved in the synthesis of simple sugars belonging to the raffinose family (Monroy *et al.* 1993; Wolfraim and Dhindsa 1993; Wolfraim *et al.* 1993; Cunningham *et al.* 2003; Haagenson *et al.* 2003a). The role of these proteins in freezing tolerance may include protecting the cell from freezing injury by maintaining cell membrane integrity, protecting cells from reactive oxygen species, increasing the solute concentration in the cell to reduce the freezing point, and increasing the osmotic potential of the cytoplasm (Castonguay *et al.* 2006). These processes potentially protect the cell from damage due to dehydration, which would mean that they could also be up-regulated under drought conditions. The objective of this study was to compare the abundance of VSPs, β-amylase, dehydrins and sucrose synthase proteins and transcripts (including a CAR gene) in taproots of two contrasting lucerne cultivars during a regrowth cycle under the influence of water deficit stress.

8.2. Materials and Methods

8.2.1. Plant tissue collection

The taproot samples from three replications of Grasslands Kaituna and SARDI 10 plants receiving 100% and 25% of the replacement water requirement described in Chapters 6 and 7 were used for this analysis. At the destructive harvests occurring at H_0, 14 days after H_0 and 35 days after H_0, the top 50 mm of the two taproots that were not frozen on dry ice (Chapter 7) were diced and frozen in liquid N. Using a mortar and pestle, this tissue was ground to a fine powder in liquid N.

8.2.2. Extraction and analysis of the taproot soluble protein pool

Soluble proteins were extracted from freeze dried taproot tissue and quantified using the methods described in Chapter 5. After quantification, protein extract was mixed one-to-one with SDS-PAGE sample loading buffer. The SDS-PAGE, gel staining

and quantification procedures used were the same as those described in Chapter 5. In this analysis optical intensity (arbitrary units) of bands corresponding to the VSPs, as well as β-amylase, were determined.

Western blotting was used to confirm that analysed bands corresponded to the VSPs and β-amylase, as well as to visualise changes in the abundance of sucrose synthase and dehydrins. Extracted protein from selected samples (2.5 µg for VSP and β-amylase, 10 µg for sucrose synthase and 20 µg for dehydrin analyses) were separated by SDS-PAGE. Western blots were created, using the procedure described in Chapter 5. All blocking, inoculation, washing and detection steps occurred using the procedures described previously (Chapter 5). Dilutions of the primary antibodies used were 1:10 000 for VSP (Cunningham and Volenec 1996), 1:1000 for sucrose synthase, 1:2000 for β-amylase (ab6617, Abcam Inc., Cambridge, MA, USA), and 1:750 for polyclonal dehydrin antibodies (PLA-100, Stressgen Bioreagents Crop, Ann Arbor, MI, USA). The dilution of the goat-anti-rabbit antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA, USA) was 1:3000.

8.2.3. RNA extraction and northern blotting

Total RNA was extracted from tissue frozen in liquid N using the modified hot phenol procedure, and its quantity, quality and integrity checked using the methods used in Chapter 5. Northern blots were created after the electrophoresis of RNA in formaldehyde agarose gels (Plate 8.1), probed with radio labelled cDNAs and washed using the procedures described in Chapter 5. The cDNA sequences used corresponded to the sequences for the genes CAR1, high molecular weight VSP, β-amylase and the sucrose synthase. Characteristics of these cDNAs are outlined in Table 5.1. Exposure and film development procedures used have been described previously (Chapter 5).

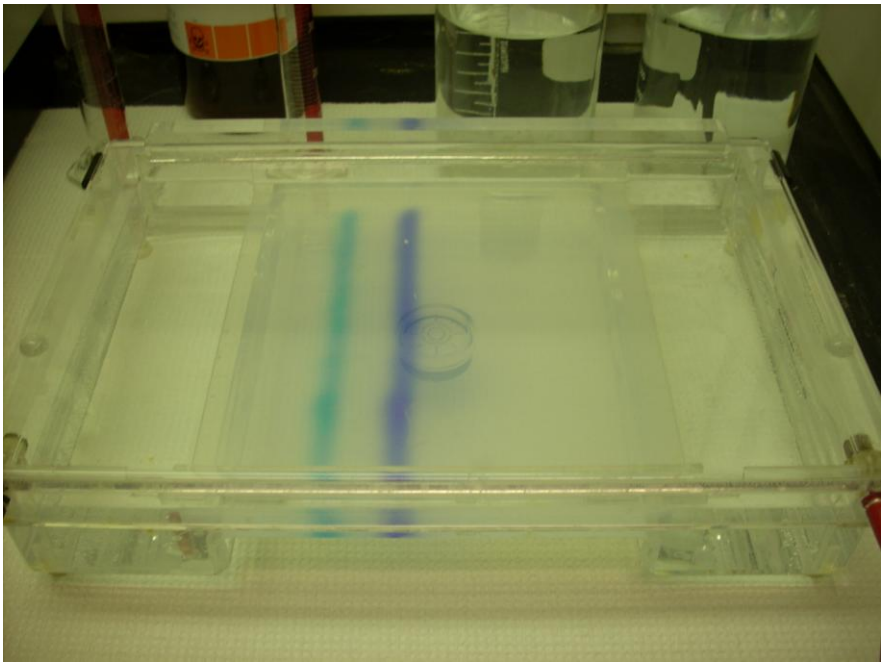

Plate 8.1. Electrophoresis of RNA in a formaldehyde agarose gel as part of the process of creating a Northern blot.

8.2.4. Quantitative reverse transcriptase polymerase chain reaction analysis of transcript abundance

For quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis 20 µg of extracted RNA was DNase digested using the Ambion DNA-free kit (Applied Biosystems, Foster City, CA, USA) to remove any genomic deoxyribonucleic acid (DNA) contamination. Following DNase digestion, RNA was re-quantified and its integrity was checked by visualisation after electrophoresis of 5 µg of DNase digested RNA in formaldehyde agarose gels. Absence of genomic DNA was confirmed by performing polymerase chain reactions (PCRs) on 0.002 µg of RNA with forward and reverse primers designed to amplify a 154 base pair fragment of the lucerne acetyl CoA carboxylase gene (5 prime to 3 prime; forward: GATCAGTGAACTTCGCAAAGTAC; reverse: GAGGGATGCTGCTACTTTGATG; Alexander *et al.* 2007; GenBank Accession L25042). Visualisation of PCR products was achieved by electrophoresis in 3% MetaPhor agarose gels stained with ethidium bromide. Synthesis of cDNA was carried out with 1 µg of DNase digested RNA using the iScript cDNA synthesis kit (Bio-Rad laboratories). Following cDNA synthesis 2 µL from each reaction was combined to create a "calibrator" sample as described by Larionov *et al.* (2005)

which was then serial diluted to create 1:5, 1:20, 1:80, 1:320 and 1:1280 dilutions generating a standard curve.

Candidate reference genes for normalisation were chosen based on a list of 79 constitutively expressed probe sets (validated using GeNorm; Vandesompele *et al.* 2002, and NormFinder; Andersen *et al.* 2004) in *Medicago truncatula* grown under drought conditions. These genes were identified using affymetrix chip experiments as part of the *M. truncatula* Gene Expression Atlas (MtGEA) Project (V.A. Benedito pers. comm. 2009). These probe sets were compared with known lucerne RNA sequences in GenBank, and 8 homologs were identified (Table 8.1). Primers were designed for these eight candidate genes, as well as for the four genes of interest. CAR1, high molecular weight VSP and β-amylase gene primers were based on the published sequences of the cDNAs used for Northern blotting (Table 5.1). Primers for sucrose synthase were designed using a lucerne sucrose synthase sequence in GenBank (Accession no. AF049487). Primers were designed with the Primer express 3.0 software (Applied Biosystems; Rozen and Skaletsky 2000). Criteria for primer design were as follows: a predicted melting temperature between 58 and 60^{o}C, an optimal primer length of 20 base pairs (bp), GC content between 30 and 80%, and an amplicon length between 50 and 150 bp. Primers were checked for possible hairpins and self-complementation using "Oligo Calc" (http://www.basic.northwestern.edu/biotools/OligoCalc.html; accessed April 20 2009; Kibbe 2007). Primer sequences that exhibited either of these properties were discarded and new primers designed and checked. Primers were custom-ordered from a commercial supplier (Eurofins MWG Operon, Huntsville, AL, USA). To confirm specificity of the primers, melting curve analysis was performed after each qPCR reaction, and PCR products were visualised using gel electrophoreses in 3% MetaPhor agarose gels stained with ethidium bromide.

Reactions were performed in 384 well plates sealed with optical adhesive covers (Micro Amp Optical PCR Plate and Micro Amp Optical Adhesive Covers, Applied Biosystems) in a 7900HT Fast Real Time PCR system (Applied Biosystems) using iTaq SYBR green Supermix with ROX (Bio-Rad Laboratories). Reactions were carried out in triplicate and contained 5 µL of SYBR green Supermix, 0.375 µL each of forward and reverse primers, 2 µL of a 1:50 dilution of template cDNA, and

2.5 µL of PCR-grade water. Each plate also contained the serial dilutions of the calibrator sample in triplicate to create a standard curve as well as negative controls for each primer pair. The reaction conditions were as follows: 50°C for two minutes, 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds followed by 60°C for one minute. Sequence detection software (SDS version 2.3; Applied Biosystems) was used to determine the quantification cycle.

Quantification cycle values and the standard curves were used to calculate the relative concentration of each gene in each sample following the standard curve method for relative quantitative PCR data processing (Larionov *et al.* 2005). Concentration values of the genes of interest were normalised using the geometric mean of the three most stably expressed reference genes as identified using GeNorm (Vandesompele *et al.* 2002). Normalized concentration values were divided by the normalised concentration values for plants of Grasslands Kaituna sampled at defoliation before the initiation of water treatments to give a relative concentration (Equation 8.1).

Equation 8.1:

$$\text{Relative concentration} = \frac{T/(R_{T1} \times R_{T2} \times R_{T3})^{1/3}}{C/(R_{C1} \times R_{C2} \times R_{C3})^{1/3}}$$

Where T is the concentration of the gene of interest in the treatment sample, C is the concentration of the gene of interest in the pre-treatment control sample and R_{T1}, R_{T2} and R_{T3} and R_{C1}, R_{C2} and R_{C3} are the concentrations of the 3 reference genes in the treatment and control samples respectively.

8.2.5. Statistical analysis

Densitometry measurements of optical intensity of selected bands from the SDS-PAGE analysis of soluble protein pool composition, and log transformed relative abundance of gene transcripts from qRT-PCR analysis, were analysed as an ANOVA of a randomised complete block design, with cultivar, water treatment and days after defoliation as factors. For densitometry measurements, when ANOVA identified a significant effect, regressions were fitted to the data. Unless otherwise stated significance was accepted when $P < 0.05$. All statistical analysis was achieved using Genstat 11th edition (VSN International Ltd, Hemel Hempstead, UK).

Table 8.1. Primer pairs (F: forward, R: reverse) of the eight reference and the four genes of interest used in the quantitative RT-PCR analysis.

Type (reference gene/ gene of interest)	Gene Name	Genbank Accession no.	†MtGEA probeset ID	Primer sequence	Predicted fragment size (bp)
Reference gene	ADP-ribosylation factor	AY466444	Mtr.8432.1.S1_at	F: GAGAGGGTCTTTACGAGGGTTTG R: TCTCATTATGCCTTGCTTGCA	64
Reference gene	Phosphoprotein phosphatase type 2A	X70399	Mtr.10878.1.S1_at	F: CCGATGTCACTCACGATCTCA R: GAAAGTGGTTTGCACTGCATGA	61
Reference gene	Histone H3	X13674	Mtr.16911.1.S1_s_at	F: GAAGCGGCCGAGGCTTA R: GAATGGCGCAGAGATTAGTATCCT	58
Reference gene	GTP-binding Protein	X79278	Mtr.17963.1.S1_at	F: GCGAAACCACACAGATTCCA R: GTGACGGAGATCTGCTTTGTTG	63
Reference gene	Translationally controlled tumour protein	X63872	Mtr.27459.1.S1_s_at	F: CGGCAAGCTCAAGGACCTT R: CCAAGCTACCATCATCATGCA	62
Reference gene	Calmodulin	X52398	Mtr.38439.1.S1_at	F: CAAGGAGCTCGGGACTGTGA R: CCTGCAATTCAGCCTCAGTTG	62
Reference gene	Elongation initiation factor	X59441	Mtr.43167.1.S1_at	F: CCGCCACTTCTGAATCAATATG R: CATCGGCCTTGGATTCAAAT	59
Reference gene	Glyceraldehyde-3-phosphate dehydrogenase	EF526095	Mtr.51186.1.S1_at	F: CAAAGCTGCTGCTCACTTGAA R: CATCTTTGCTGGGAGCAGAAA	65
Gene of interest	CAR1	AF072932	‡NA	F: AGCAGCAGCAGTGACAGTGATT R: CATATGACTCCATACACCTCTCAACA	73
Gene of interest	High molecular weight VSP	AF530579	NA	F: CCCGAAATAAAACACTGTCAACAA R: CCTACTGCACCTCCGATCGA	65
Gene of interest	β-amylase	AF026217	NA	F: GCAGCTGCAGCGAAAGCT R: TTGTACGTGCCAGCGTCATC	62
Gene of interest	Sucrose synthase	AF049487	NA	F: ACTGGTGTCTATGGCTTCTGGAA R: GATAGCGGCGGCTCTCAAG	64

†MtGEA: *Medicago truncatula* Gene Expression Atlas; ‡NA: not applicable

8.3. Results

8.3.1. Taproot soluble protein pool composition

The composition of the soluble protein pool in taproots of both cultivars was influenced by water deficit and days after defoliation (Figure 8.1). The optical intensity of bands corresponding to β-amylase and the high and middle molecular weight VSPs were affected ($P < 0.001$) by days after defoliation. There was a cultivar by days after defoliation interaction on the optical intensity of bands corresponding to the low molecular weight VSP. Bands corresponding to β-amylase and the high and middle molecular weight VSPs showed a linear increase in optical intensity when compared with days after defoliation (Table 8.2). For the low molecular weight VSP in Grasslands Kaituna, the effect of day after defoliation on optical intensity was quadratic, while, for SARDI 10, it was linear (Table 8.2). Averaged across both cultivars and all harvests, optical intensity of bands corresponding to both the middle and low molecular weight VSPs was greater in plants exposed to water deficit, while there were no differences in the optical intensity of bands corresponding to the high molecular weight VSP and β-amylase when plants receiving the full water requirement were compared to those receiving 25% of the replacement water requirement.

Western blotting confirmed the increase in β-amylase and the high, middle and low molecular weight VSPs throughout the regrowth cycle (Figure 8.1). Two β-amylase bands were detected on Western blots of Grasslands Kaituna when compared to SARDI 10 under both watering conditions (Figure 8.1C). Sucrose synthase abundance increased between 14 and 21 days after defoliation under both watering treatments, and dehydrins were present in taproots of plants that received 25% of the replacement water requirement towards the end of the regrowth period (Figure 8.1).

Table 8.2. Regressions explaining changes in the optical intensity (O) of bands from SDS-PAGE analysis corresponding to β-amylase and the high, middle and low molecular weight VSPs when compared with days of regrowth (D). Data for β-amylase and the high and middle molecular weight VSPs were averaged over both cultivars and water treatments. Data for the low molecular weight VSP in each cultivar was averaged over both water treatments.

Protein	Regression model	P value	R^2
β-amylase	$O = 13.8 \times D + 527$	<0.001	97
High molecular weight VSP	$O = 16.9 \times D + 639$	0.001	94
Middle molecular weight VSP	$O = 23.1 \times D + 583$	0.008	82
Grasslands Kaituna: low molecular weight VSP	$O = 1.0 \times D^2 - 8.7 \times D + 569$	0.004	96
SARDI 10: low molecular weight VSP	$O = 10.9 \times D + 746$	0.046	59

8.3.2. Northern analysis of transcript abundance

In taproots of plants from both cultivars grown under both watering regimes transcripts encoding the high molecular weight VSP increased between 14 and 35 days of regrowth (Figure 8.2). Transcript abundance of β-amylase increased between 14 and 35 days of regrowth in taproots of Grasslands Kaituna. When compared to SARDI 10, Grasslands Kaituna plants that were grown under a water deficit had more β-amylase transcripts at both 14 and 35 days of regrowth (Figure 8.2). Sucrose synthase transcript levels were elevated slightly at 14 and 35 days of regrowth in taproots of Grasslands Kaituna that were grown under a water deficit, while in taproots of SARDI 10, abundance of transcripts did not change (Figure 8.2). Transcripts of CAR1 increased in abundance in taproots of Grassland Kaituna plants that were grown under a water deficit when compared to fully watered plants. CAR1 expression in plants of SARDI 10 was unaffected by water deficit at either 14 or 35 days after defoliation (Figure 8.2).

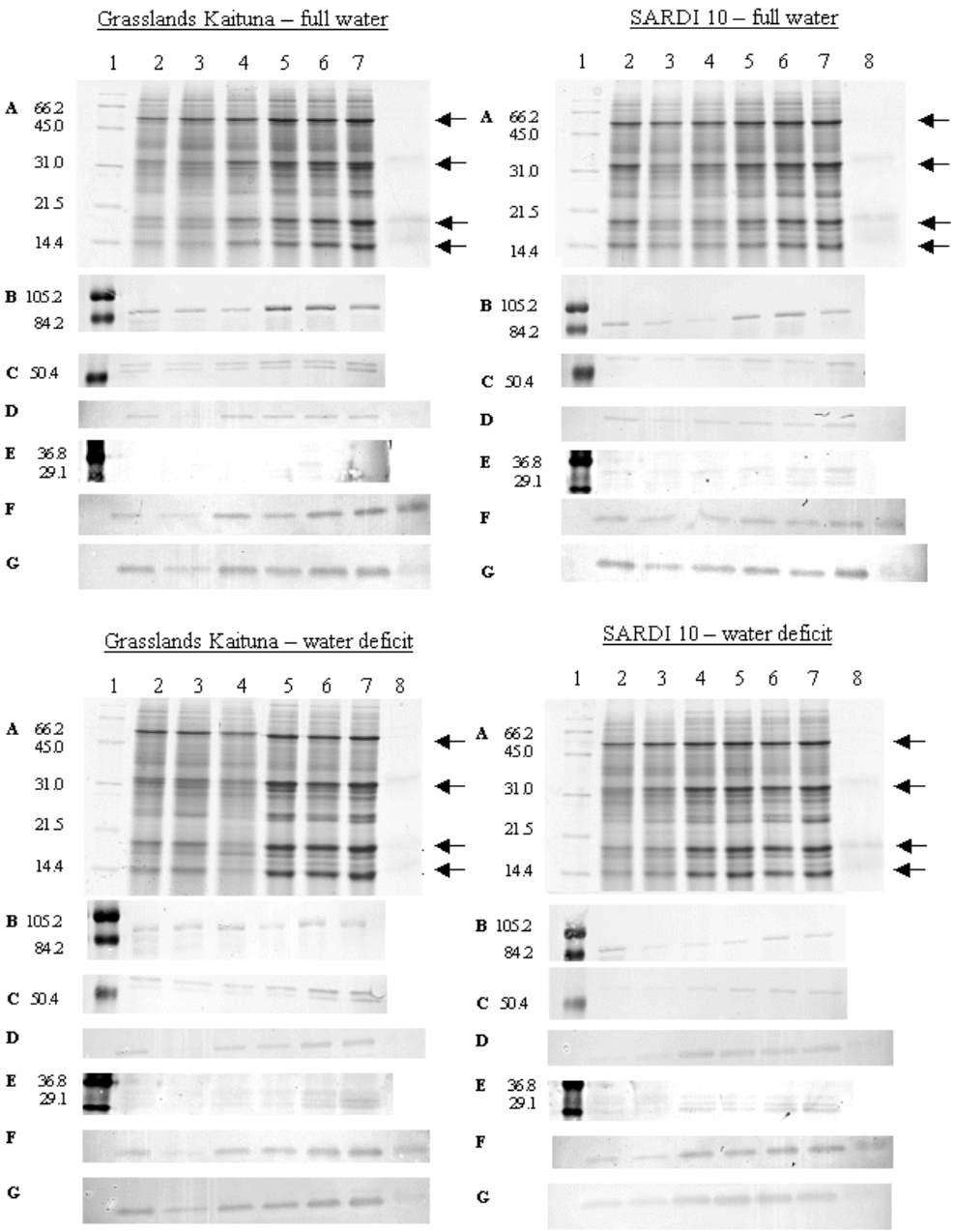

Figure 8.1. SDS-PAGE analysis (A) and Western blot analysis using antibodies raised to sucrose synthase (B), β-amylase (C), dehydrin (E), middle molecular weight VSP (F), and low molecular weight VSP (G) using soluble protein extracted from taproots of Grasslands Kaituna and SARDI 10 lucerne receiving 100% or 25% water and sampled 0 (Lane 2), 7 (Lane 3), 14 (Lane 4), 21 (Lane 5), 28 (Lane 6), and 35 (Lane 7) days after defoliation. In 'D' the Western blot shows the cross specificity of antibodies raised to the low molecular weight VSP with the high molecular weight VSP. Lane 1 on the SDS-PAGE gels, as well as on Western blots B, C and E, are molecular weight standards. Lane 8 on the gels and Western blots D, F and G contains the purified lucerne VSPs. Arrows highlight the bands for which optical intensity was determined, and the numerals down the right are the size (in kD) of the molecular weight standards.

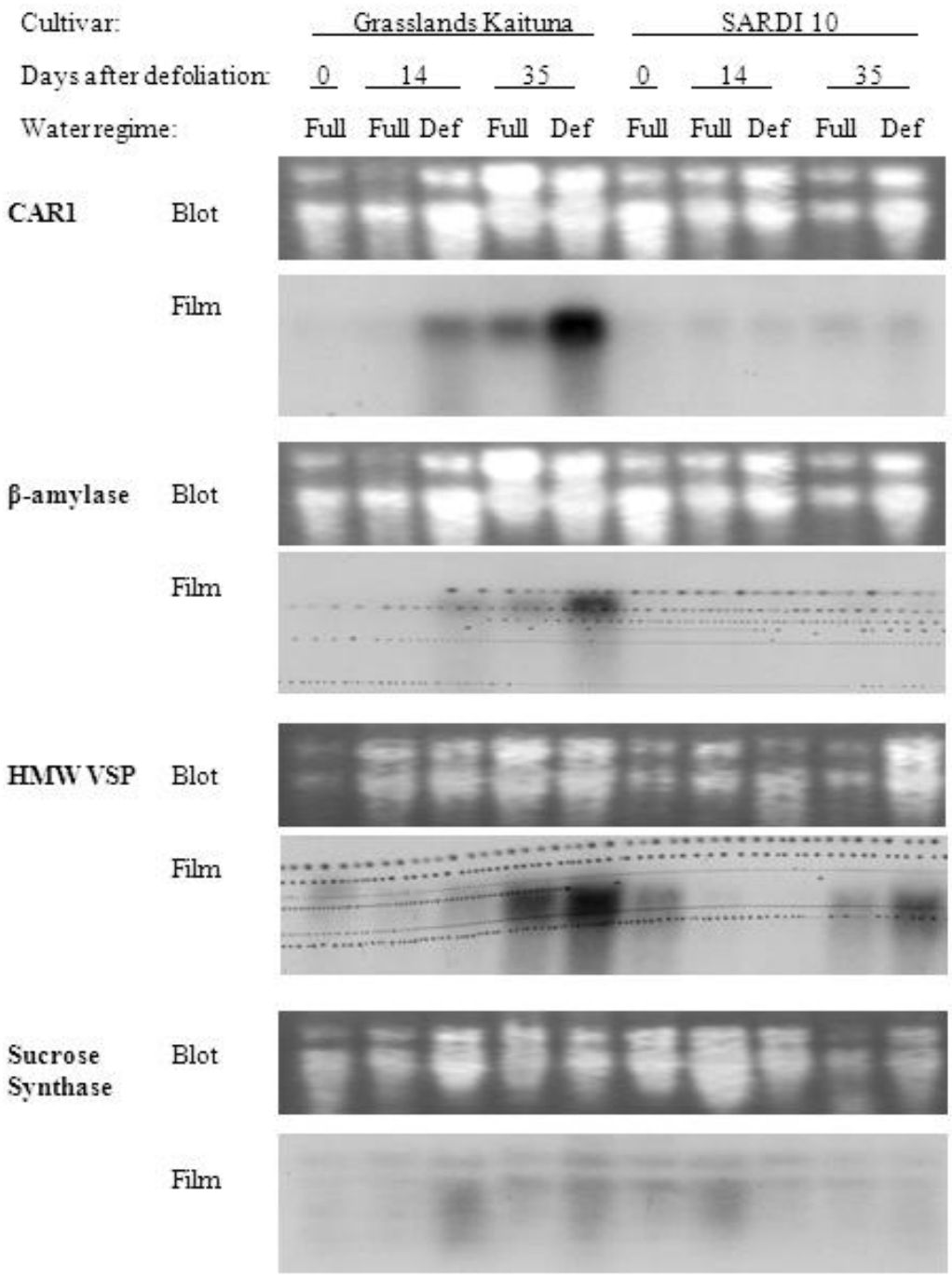

Figure 8.2. Northern analysis of transcript abundance of the genes CAR1, β-amylase, high molecular weight (HMW) VSP, and sucrose synthase in the taproots of Grasslands Kaituna and SARDI 10 lucerne regrowing under either fully watered (Full) or water deficit (Def) conditions.

8.3.3. Quantitative RT-PCR analysis of transcript abundance

The primers used in quantitative RT-PCR analysis (Table 8.1) were specific to the genes for which they were designed. Melting curve analysis showed only one peak present for each primer pair (Figure 8.3) and analysis of reaction products in MetaPhor agarose gels showed only one band present for each primer pair (Figure 8.4). These bands approximated the size of the predicted amplified fragment based on gene sequence information. All transcripts were moderately abundant (median quantification cycles between 28 and 19) except for the high molecular weight VSP gene, which had a median quantification cycle value of 18 (Figure 8.5). Intra-sample variation was greater in all the genes of interest when compared to the reference genes (Figure 8.5).

Pair-wise variation analysis with GeNorm showed that three reference genes would be required for adequate normalization of the abundance of transcripts from the genes of interest in this sample set (Figure 8.6). The three candidate reference genes with the lowest expression stability, as determined by GeNorm, were the GTP binding protein, elongation initiation factor and ADP- ribosylation factor (Figure 8.7).

There was a cultivar by water treatment by days after defoliation interaction effect on both the relative abundance of CAR1 and β-amylase transcripts. Transcripts of CAR1 were most abundant in the taproots of water-stressed Grasslands Kaituna plants 35 days after defoliation (Figure 8.8). Transcript abundance of β-amylase was greatest in taproots of Grasslands Kaituna at 35 days of regrowth in both water regimes (Figure 8.9). The relative abundance of transcripts encoding for the high molecular weight VSP was affected ($P < 0.001$) by sampling, increasing from 1.3 times greater than the pre-defoliation control at 14 days of regrowth to 4.7 times greater than the pre-defoliation control at 35 days of regrowth. There was no effect of cultivar and no interaction between cultivar and watering treatment on VSP abundance. Sucrose synthase abundance was not affected by watering treatment, cultivar or harvest, nor was there an interaction effect between these factors.

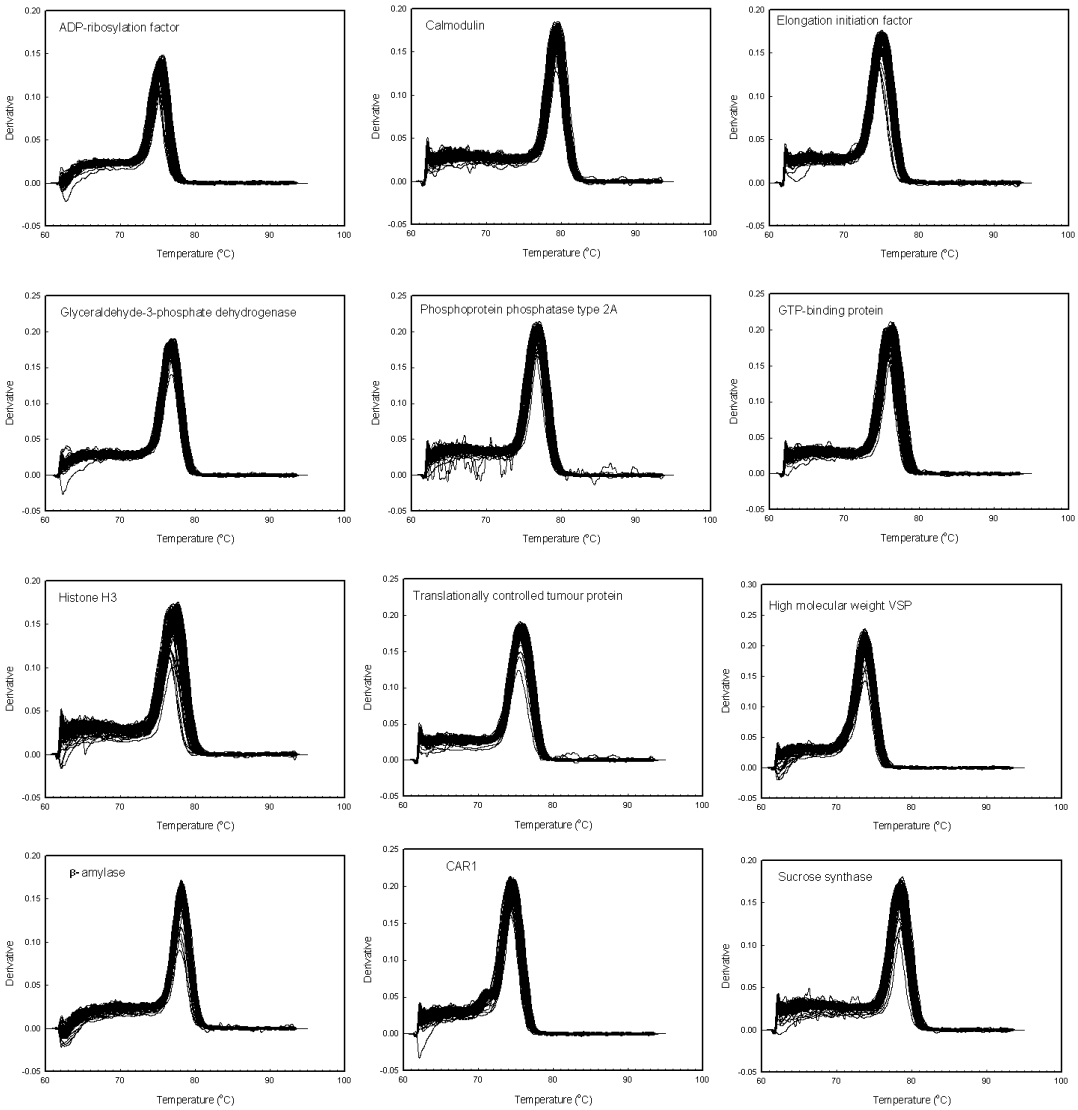

Figure 8.3. Melting curves of PCR products from amplifications using primers designed to eight candidate reference genes (ADP-ribosylation factor, calmodulin, elongation initiation factor, glyceraldehyde-3-phosphate dehydrogenase, phosphoprotein phosphatase type 2A, GTP-binding protein, histone H3, and translationally controlled tumour protein) and the four genes of interest (high molecular weight VSP, β-amylase, CAR1, and sucrose synthase).

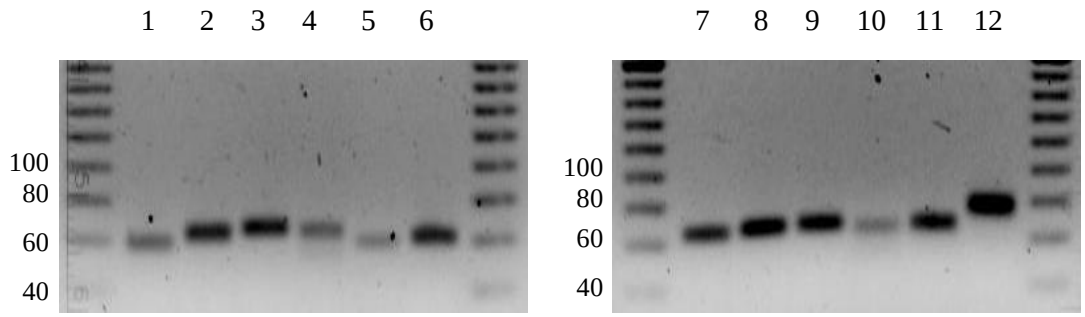

Figure 8.4. Resolution using 3% (w/v) MetaPhor agarose gels of reaction products from reactions using primers designed for histone H3 (Lane 1), calmodulin (Lane 2), glyceraldehyde-3-phosphate dehydrogenase (Lane 3), ADP-ribosylation factor (Lane 4), elongation initiation factor (Lane 5), phosphoprotein phosphatase type 2A (Lane 6), translationally controlled tumour protein (Lane 7), GTP-binding protein (Lane 8), high molecular weight VSP (Lane 9), β-amylase (Lane 10), sucrose synthase (Lane 11), and CAR1 (Lane 12). A 20 base pair molecular ruler is shown on either side of each gel. Numerals to the right of the gels show the size of markers in base pairs.

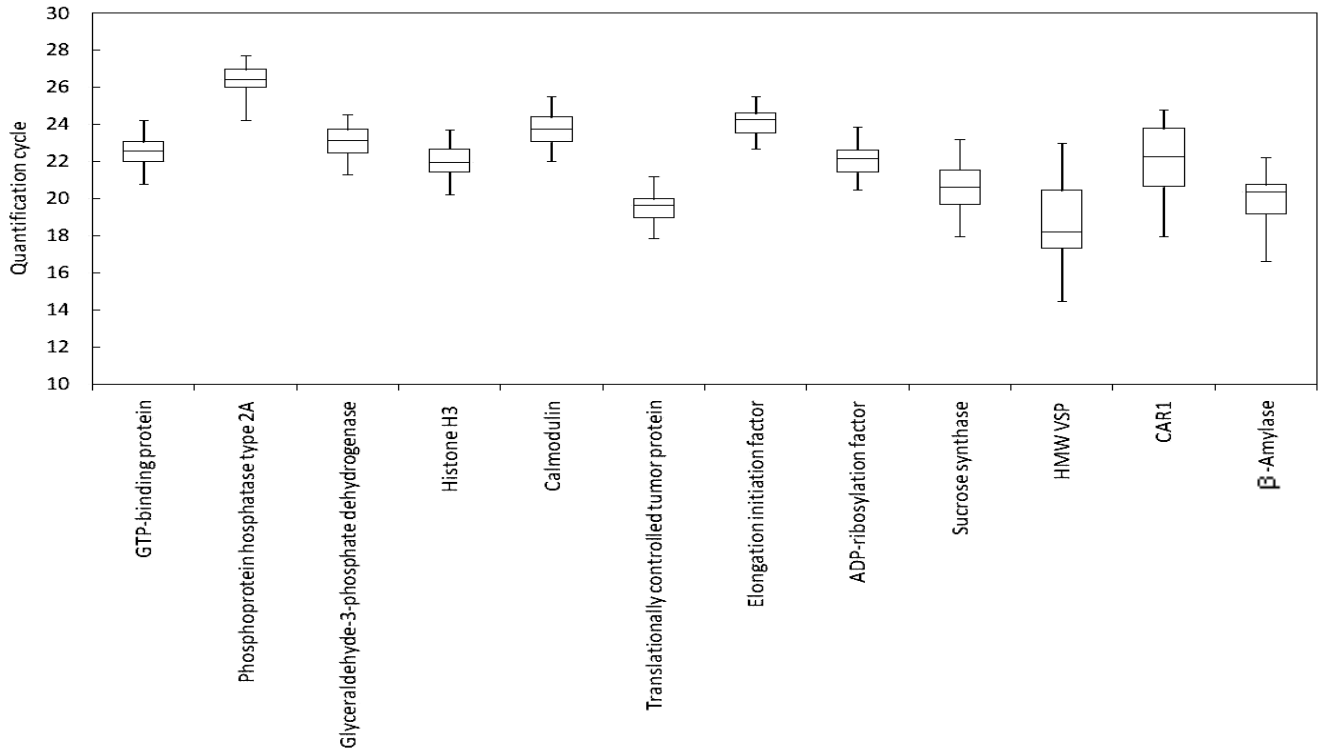

Figure 8.5. Box and whisker plots showing variability in the quantification cycle of candidate reference genes (GTP-binding protein, phosphoprotein phosphatase type 2A, glyceraldehyde-3-phosphate dehydrogenase, histone H3, calmodulin, translationally controlled tumour protein, elongation initiation factor, and ADP-ribosylation factor) and genes of interest (sucrose synthase, high molecular weight VSP, CAR1, and β-amylase) and in the quantitative RT-PCR analyses. Lines represent the median, boxes represent the 25th and 75th percentiles, and whiskers represent the range of each gene of interest. Statistics were calculated from 30 biologically independent samples.

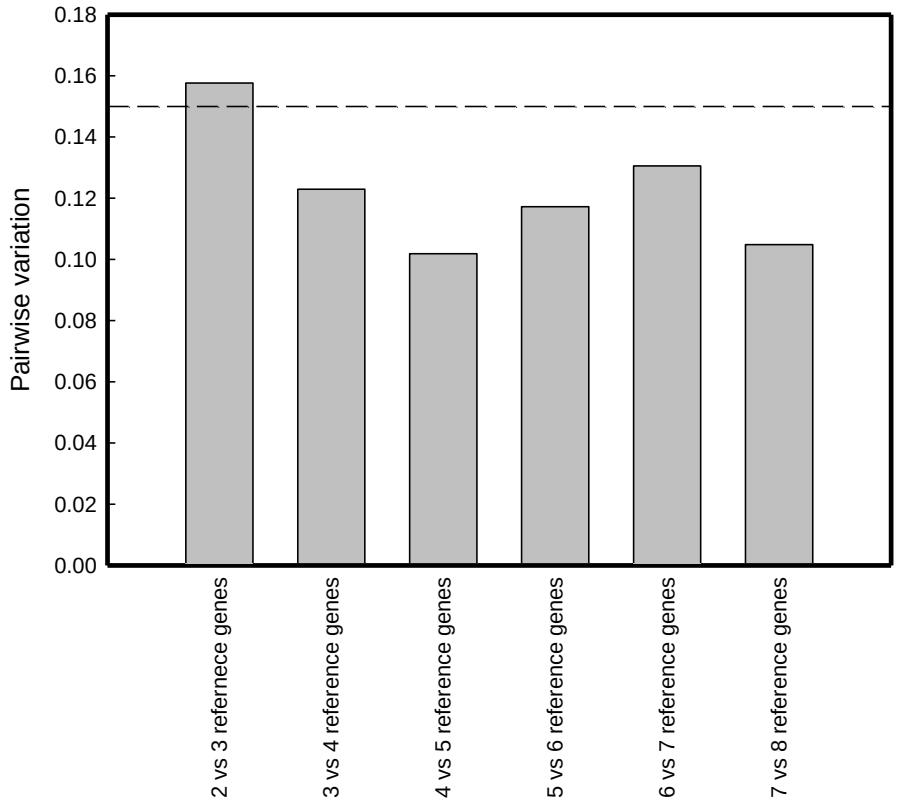

Figure 8.6. Pair-wise variation analysis from GeNorm to determine the optimal number of reference genes required for the adequate normalisation of quantitative RT-PCR results from taproots of Grasslands Kaituna or SARDI 10 lucerne regrowing under drought conditions. The horizontal broken line shows the 0.15 cut off in pair wise variation suggested in Vandesompele *et al.* (2002) as a threshold to determine the optimum number of reference genes.

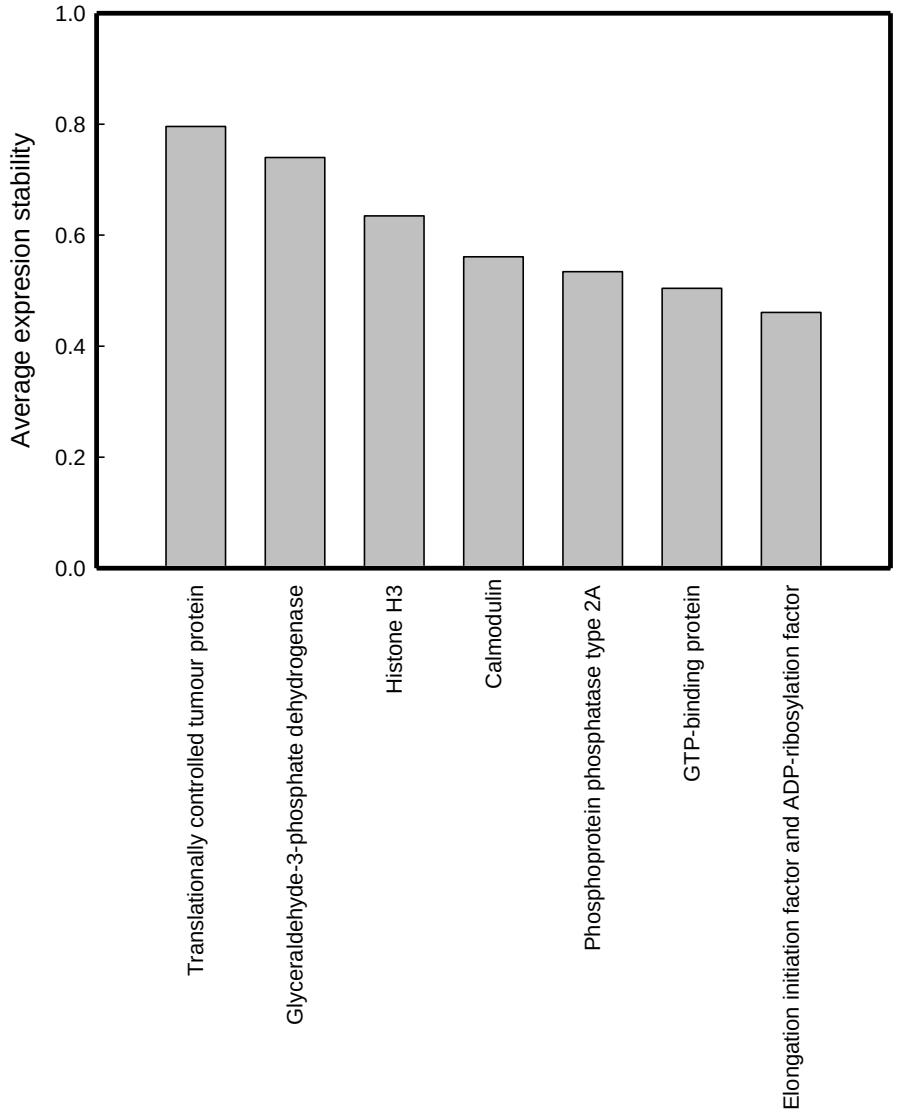

Figure 8.7. Average expression stability calculated by GeNorm from quantitative RT-PCR results using primers designed for 8 candidate reference genes in taproots of Grasslands Kaituna or SARDI 10 lucerne regrowing under fully watered or drought conditions.

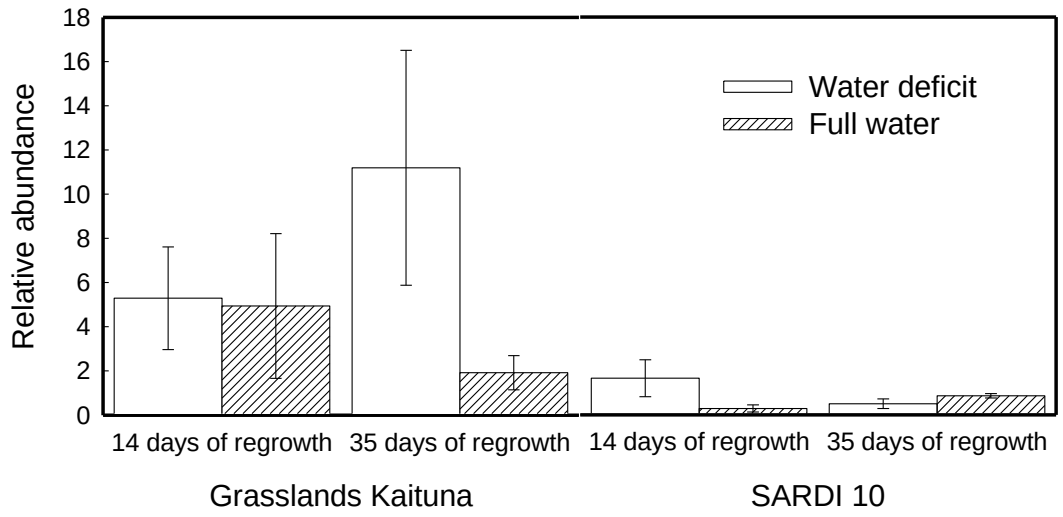

Figure 8.8. Relative abundance (determined by quantitative RT-PCR) of lucerne CAR1 transcripts in the RNA pool from Grasslands Kaituna or SARDI 10 taproots at 14 and 35 days of regrowth under either fully watered or water deficit conditions. Abundance is relative to transcript levels in fully watered Grasslands Kaituna lucerne at defoliation and prior to the initiation of water treatments. Error bars represent the standard errors (n = 3).

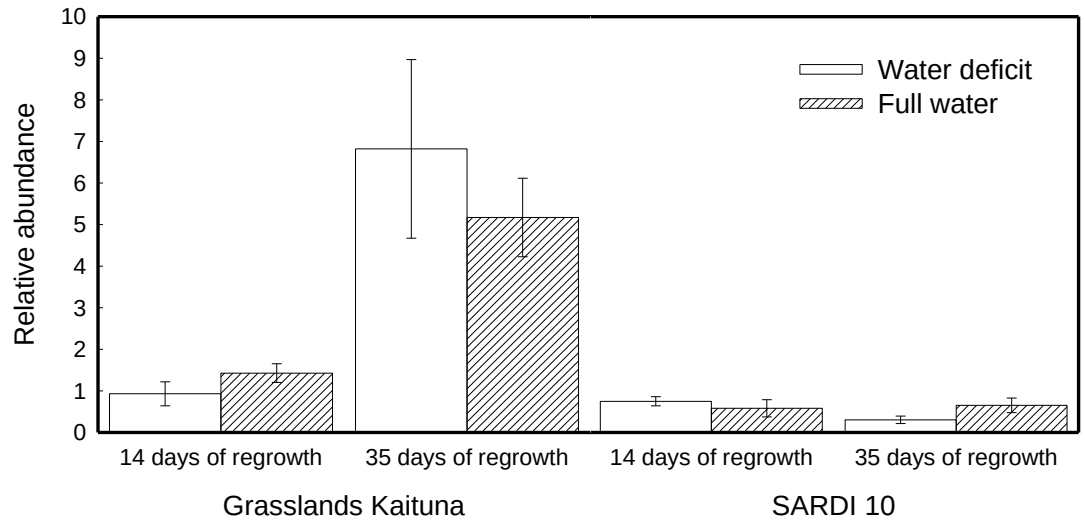

Figure 8.9. Relative abundance (determined by quantitative RT-PCR) of lucerne β-amylase transcripts in the RNA pool from Grasslands Kaituna or SARDI 10 taproots at 14 and 35 days of regrowth under either fully watered or water deficit conditions. Abundance is relative to transcript levels in fully watered Grasslands Kaituna lucerne at defoliation and prior to the initiation of water treatments. Error bars represent the standard errors (n = 3).

8.4. Discussion

There was no difference in the pattern of VSP accumulation in either cultivar when plants were grown under a water deficit. Plants regrowing under a water deficit contained a greater abundance of low and middle molecular weight VSPs compared to plants grown under fully watered conditions. The absence of an increase in the high molecular weight VSP with water deficit was unexpected as previous reports have noted that all three lucerne VSPs respond similarly to environmental stimuli (Kalengamaliro *et al.* 1997; Cunningham *et al.* 1998; Haagenson *et al.* 2003b). Potentially, the method used for determining optical intensity may not have been sensitive enough to detect differences in the high molecular weight VSP, as flat bed scanners using white light are not as sensitive as commercially available densitometers (Vincent *et al.* 1997; Tan *et al.* 2007). While this is the first examination of VSP abundance throughout a regrowth cycle for lucerne grown under drought conditions, greater abundance of VSPs in the taproots of drought-stressed lucerne compared to fully watered plants at the end of a regrowth cycle have been observed previously (Erice *et al.* 2007).

Abundance of β-amylase increased throughout the regrowth period. It has previously been shown that β-amylase acts as a VSP in lucerne (Gana *et al.* 1998). There was no affect of water deficit on the abundance of VSP transcripts in either cultivar or for β-amylase in Grasslands Kaituna. This, combined with the same pattern of changes in the abundance of VSPs and β-amylase, suggests that VSPs do not play an active role in the adaptation of lucerne to drought conditions or salvage N from tissues under stress. Both these roles would require an increase in VSP gene transcript abundance and an altered accumulation pattern of VSP in the soluble protein pool in drought-stressed plants. Shoot growth is considerably reduced in lucerne regrowing during a water deficit (Brown and Tanner 1983; Carter and Sheaffer 1983b; Durand *et al.* 1989). As N accumulation through fixation is more sensitive to drought than shoot growth (Irigoyen *et al.* 1992b) the reduced demand for VSPs to supply N for the formation of new shoots explains the increase in taproot VSP abundance that is associated with drought stress.

The difference between cultivars in the abundance of the β-amylase transcript was unexpected. Previous examination of β-amylase transcript abundance has shown

that across cultivars varying in winter dormancy class, β-amylase responds in the same manner as the VSPs to environmental stimuli during autumn and winter (Gana *et al.* 1998). However, previous studies of β-amylase expression during regrowth under summer conditions have focused on semi winter-dormant and winter-active cultivars, with no assessment of highly winter-active cultivars (Gana *et al.* 1998; Berg *et al.* 2009). Previous reports, and the results from this study, highlight a potential winter activity class by environment interaction on β-amylase transcript abundance, and further studies of this interaction are warranted.

While this was the first study of the expression of CAR1 in the taproots of lucerne grown under drought conditions, the up-regulation of this gene in Grasslands Kaituna, but not SARDI 10, agrees with previously reported genotype by environment stimuli interactions (Haagenson *et al.* 2003a). The expression of winter acclimation/cold tolerance genes during drought stress was not unexpected, as both environmental stresses (freezing and drought) involve a considerable level of cell dehydration (Castonguay *et al.* 2006).

In Tasmania, when grown in water limited environments, Grasslands Kaituna has greater annual DM yield compared to SARDI 10, while no yield differences between these two cultivars exist when plants are irrigated (Chapter 4). An up-regulation of CAR1 and other cold tolerance genes in response to abiotic stress may explain the better performance of Grasslands Kaituna compared to SARDI 10 when grown without irrigation, as well as, the better adaptation of winter dormant genotypes to unfavourable environments when compared to highly winter active genotypes (Chapter 3).

One of the most well-studied protein families associated with drought tolerance are the dehydrins (Close 1996). These proteins have a role in maintaining membrane stability as cells dehydrate (Close 1997). Searching lucerne nucleotide sequences in GenBank, using a 15 amino acid residue sequence that is highly conserved across dehydrins from a broad range of plant species (Close 1997), identified six nucleotide entries. Of these sequences, three encode for punitive proteins between 30 and 33.5 kD, five are associated with peer-reviewed publications (Wolfraim and Dhindsa 1993; Wolfraim *et al.* 1993; Ivashuta *et al.* 2002; Pennycooke *et al.* 2008; Remus-borel *et al.* 2010), and their annotations suggest up-

regulation with cold temperatures. As such, the presence of dehydrins in both cultivars grown under a water deficit does not support our initial hypothesis that freezing tolerance genes confer an advantage to winter dormant lucerne cultivars grown under drought conditions. Clearly the molecular and biochemical processes responsible for freezing and drought tolerance in lucerne are complex, and a greater understanding of the underlying molecular mechanisms of these processes would benefit both areas of research. If there is a link between freezing tolerance and drought tolerance, germplasms with high freezing tolerance and high rates of fall growth that have been identified by Brouwer *et al.* (2000) and Brummer *et al.* (2000) might be a source of lucerne germplasm capable of maintaining growth under water-limited conditions.

Sucrose synthase abundance increased between 14 and 21 days after defoliation and this increase was most apparent in the fully watered plants. However, an increase in transcripts encoding for sucrose synthase was not detected during this time period by quantitative RT-PCR analysis, and Northern analysis only showed a faint increase in sucrose synthase transcript abundance. The timing of sampling may have prevented the identification of changes in the regulation of the sucrose synthase transcript as Berg *et al.* (2009) showed that the initial decrease in this gene's transcripts after defoliation continued until 14 days after defoliation.

In conclusion, lucerne VSPs have a limited role in the plant's cellular adaption to water deficit, while genes associated with freezing tolerance may confer an advantage to the plant's adaptation to drought. The increase in transcripts of the gene associated with freezing tolerance suggests a mechanism explaining the improved performance of the more winter dormant cultivars under dryland conditions observed in Chapter 4. Further studies into the regulation of cold tolerance genes in lucerne grown under drought conditions seem warranted.

CHAPTER 9

General discussion

The wide genetic diversity within the lucerne species, coupled with variation in environment and farming practices, leads to genotype by environment interaction effects on plant yield and persistence. Because of this diversity, an evaluation of genotype by environment interactions in the cool temperate dairy regions of Australia is required to determine if they can be exploited through cultivar selection and genotype-specific management practices (Cooper *et al.* 2006; Annicchiarico 2009). The complex nature of genotype by environment interactions means that, to fully understand them and ensure that they will be repeatable; an evaluation must be undertaken at the crop, plant and cellular level.

An evaluation of genotype by environment interactions at the crop level was undertaken through an analysis of lucerne cultivar and experimental line testing experiments in two cool temperate environments. To determine if the genotype by environmental interactions identified were repeatable with currently available cultivars, and to investigate genotype by environment interactions under dryland conditions, a field experiment was established using four contrasting locally available lucerne cultivars. Glasshouse experimentation was used to further evaluate differences between two of these four cultivars at a plant and cellular level.

The results of this thesis have shown that in the cool temperate regions of Australia there are significant genotype by environment interactions that affect the complex and quantitative traits of yield and persistence in lucerne and that this interaction is repeatable as defined by Cooper *et al.* (2006). This is despite the large degree of genetic diversity present within lucerne cultivars which would typically mask the expression of repeatable genotype by environmental interactions (Annicchiarico 2009). No genotype by environmental interaction was identified for the forage quality parameters of plant maturity at harvest or leaf to stem ratio. This

shows that in the cool temperate regions of Australia, complex traits are not always influenced by a genotype by environmental interaction.

In the cool temperate dairy regions of Australia, highly winter-active genotypes and winter-dormant genotypes are best adapted to high yield potential environments and low yield potential environments, respectively. Irrigation was a major management input determining yield potential and yield differences between cultivars. As such, winter-dormant cultivars of lucerne are recommended for growing in dryland environments, while an irrigated environment is required to obtain maximum productivity from highly winter-active cultivars. The genotype by environment interactions outlined in this thesis are consistent with cultivar recommendations in subtropical Queensland. In those regions, winter-dormant cultivars are recommended for low input extensive pastoral enterprises, and highly winter-active cultivars are recommended for intensive irrigated hay production (Lloyd *et al.* 2002). This consistency between the sub-tropical and cool temperate climatic regions of Australia suggests that the genotype by environmental interactions observed in the field experiments described in this thesis will be repeatable in a predictable manner across a broad range of climate types. Winter-dormant genotypes of lucerne clearly have an important role to play in pastoral and agricultural enterprises in environments where there is no requirement to avoid damage by freezing winter temperatures through winter dormancy.

While genotype by environment interactions were clearly evident for crop production, there was no genotype by environment influence on the relative contribution of each yield component to yield. Mass per shoot consistently had a greater contribution to yield than any other yield component. Mass per shoot has been identified as the main driver of yield, when determining the yield response to differing levels of P and K fertiliser (Berg *et al.* 2005; 2007; 2009). An analysis of shoot development under a range of water deficits identified that the total number of shoots per plant was determined within the first seven days after defoliation, while mass per shoot increased through the regrowth period. The only time that the number of shoots per plant is affected by water deficit is when the water deficit was imposed during the early vegetative stages of regrowth, and exposing lucerne to a

water deficit later in the regrowth period has no effect on shoots per plant (Perry and Larson 1974). The static nature of shoots per plant during the majority of the regrowth period means that mass per shoot is the yield component that is modified in response to environmental stimuli, and therefore has the greatest impact on yield. Agreement between this thesis and previously published reports investigating yield components (Berg *et al.* 2005; 2007; 2009), shows that this response will hold across a broad range of genotypes, environments, and management practices. This has implications for both crop management and breeding. To efficiently increase yield, crop management practices need to focus on strategies that increase mass per shoot. To meet the objective of increasing yield potential lucerne breeding programs should select for increased mass per shoot under plant densities reflecting commercial crops. The absence of genotype by environmental interaction on the relative contribution of each yield component to yield means that the selection for increased mass per shoot should be an effective method of increasing yield across a broad range of environments. This presents a possible solution for dealing with the hindrance that genotype by environmental interactions place on breeding programs (Annicchiarico 2009). The thorough understanding of the physiological processes underpinning genotype by environmental interactions developed through this thesis will enable the development of breeding programs that are capable of exploiting genotype by environmental interactions in a cool temperate climate.

When lucerne was exposed to a water deficit, the abundance of taproot VSPs increased, while optimum rates of photosynthesis were maintained up until leaf senescence occurred. However, no differences between the contrasting lucerne cultivars were identified that could explain the improved performance of Grasslands Kaituna under dryland conditions that was observed in the field experiments. This was unexpected, as previous evaluations of plant reserve storage patterns have shown differences between contrasting cultivars and their response to environmental and management stimuli (Avice *et al.* 1997b; Kalengamaliro *et al.* 1997; Gana *et al.* 1998). In the current studies, the severity of the imposed water deficits may have prevented genotypes from exhibiting their subtle differences in reserve storage.

The abundance of RNA transcripts of genes associated with freezing tolerance during autumn was found to be influenced by genotype and soil moisture availability over summer. With exposure to a water deficit, the abundance of RNA transcripts for the freezing tolerance gene CAR1 increased in Grasslands Kaituna but not in SARDI 10. Freezing and drought stress share many similarities, in that they both lead to considerable levels of cell dehydration (Castonguay *et al.* 2006). It could be expected that many of the cellular biochemical pathways and the tolerance mechanisms for freezing tolerance and cold acclimation are similar to drought acclimation and tolerance. The ability of more winter-dormant cultivars of lucerne to make these cellular changes in preparation for freezing conditions (Paquin and Pelletier 1980; Dhont *et al.* 2006b), may also convey an increased tolerance to drought conditions. This hypothesis is supported by the association between the increased abundance of RNA transcripts of the CAR1 gene in taproots of Grasslands Kaituna with the maintenance of a more favourable shoot water potential compared to SARDI 10 under a water deficit and the better performance of Grasslands Kaituna compared to SARDI 10 under dryland conditions in the field.

The association between the ability of winter-dormant cultivars of lucerne to prepare for freezing conditions and their ability to adapt to water deficits has implication for both lucerne management and breeding. Freezing tolerant genotypes may provide a new source of drought tolerant germplasm to lucerne breeding programs. Furthermore, the partial separation of the winter dormancy trait from the freezing tolerance trait achieved by Brouwer *et al.* (2000) and Brummer *et al.* (2000), presents a possibility to develop moderately winter-active cultivars that possess levels of drought tolerance similar to winter-dormant cultivars. This would take advantage of the genotype by environmental interactions described within this thesis within a breeding program as described by Annicchiarico (2009). These cultivars would be particularly useful under dryland conditions in the cool temperate regions of Australia.

The insights gained into the water deficit induced genotype by environmental interactions of key physiological processors enables possible management practices that maximise crop production under water limiting conditions to be proposed. The

increase in the abundance of VSPs in the taproots of lucerne plants exposed to drought, and their role in supplying N for the formation of new N rich tissues during regrowth (Volenec *et al.* 1996; Avice *et al.* 1997b), suggests that shoot growth of drought stressed plants would be enhanced following their release from water deficits resulting in compensatory growth. However, no increase in growth was observed following the cessation of summer drought in the field. Delays in the resumption of growth following cessation of drought have been observed by Metochis and Orphanos (1981) and Halim *et al.* (1989a). The increased abundance of RNA transcripts of cold acclimation genes with exposure to drought and the effect of summer drought on the abundance of these transcripts during autumn suggests that drought-induced dormancy is not fully broken by correcting a soil water deficit. A carryover of drought-induced dormancy into autumn would explain the delayed response to watering after drought described in this thesis and in previously published reports (Metochis and Orphanos 1981; Halim *et al.* 1989a). Shoots have been identified as the plant organ responsible for the initiation of winter dormancy in lucerne (Heichel and Henjum 1990) and so the removal of shoots by defoliation may be required to fully break plants out of drought dormancy and capture any potential compensatory growth.

Through an integration of agronomic, physiological, biochemical and molecular research techniques, this thesis has identified how genotype by environmental interactions affect lucerne at a cellular, plant and whole crop level. This has created a greater depth of knowledge to support the development of better management techniques and breeding methods to increase the yield and environmental adaption of lucerne to cool temperate environments as well as to other climatic regions.

This thesis has provided evidence that genotype by environment interactions do affect lucerne yield and persistence in the cool temperate dairy regions of Australia. Specifically, the more winter-dormant genotypes of lucerne are adapted to low yield potential dryland environments, while highly winter-active genotypes are best suited to high yield potential irrigated environments. This genotype by environmental interaction is repeatable and as such can be exploited through cultivar selection. The

ability of winter-dormant cultivars to adapt to dryland conditions appears to be associated with an increase in the abundance of RNA transcripts of genes typically associated with freezing tolerance. At a cellular level these genes may also convey tolerance to drought stress. While these processes are not yet fully understood, they do present a possible source of novel drought tolerant germplasm for the inclusion into lucerne breeding programs.

CHAPTER 10

Bibliography

Ahlgren GH (1949) 'Forage crops.' (McGraw-Hill Book Company: New York).

Al-Hamdani S, Todd GW (1990) Effect of temperature regimes on photosynthesis, respiration, and growth in alfalfa. *Proceedings of the Oklahoma Academy of Science* **70**, 1-4.

Alexander TW, Reuter T, McAllister TA (2007) Qualitative and quantitative polymerase chain reaction assays for an alfalfa (*Medicago sativa*)-specific reference gene to use in monitoring transgenic cultivars. *Journal of Agricultural and Food Chemistry* **55**, 2918 - 2922.

Allen RG, Pereira LS, Raes D, Smith M (1998) 'Crop evapotranspiration - Guidelines for computing crop water requirements - FAO Irrigation and drainage paper 56.' (Food and Agriculture Organization of the United Nations: Rome, Italy).

Alva AK, Lanyon LE, Leath KT (1985) Excess soil water and *Phytophthora* root rot stresses of *Phytophthora* root rot sensitive and resistant alfalfa cultivars. *Agronomy Journal* **77**, 437-442.

Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research* **64**, 5245-5250.

Annicchiarico P, Piano E (2005) Use of artificial environments to reproduce and exploit genotype x location interaction for lucerne in northern Italy. *Theoretical and Applied Genetics* **110**, 219-227.

Annicchiarico P (2009) Coping with and exploiting genotype-by-environment interactions. In 'Plant breeding and farmer participation'. (Eds S Ceccarelli, EP Guimarães, E Weltizien) pp. 519-564. (Food and Agriculture Organization of the United Nations (FAO): Rome).

Antolin MC, Sanchez-Diaz M (1993) Effects of temporary droughts on photosynthesis of alfalfa plants. *Journal of Experimental Botany* **44**, 1341-1349.

Aranjuelo I, Irigoyen JJ, Perez P, Martinez-Carrasco R, Sanchez-Diaz M (2006) Response of nodulated alfalfa to water supply, temperature and elevated CO_2: productivity and water relations. *Environmental and Experimental Botany* **55**, 130-141.

Aranjuelo I, Irigoyen JJ, Sanchez-Diaz M (2007) Effect of elevated temperature and water availability on CO_2 exchange and nitrogen fixation of nodulated alfalfa plants. *Environmental and Experimental Botany* **59**, 99-108.

Aranjuelo I, Perez P, Hernandez L, Irigoyen JJ, Zita G, Martinez-Carrasco R, Sanchez-Diaz M (2005) The response of nodulated alfalfa to water supply, temperature and elevated CO_2: photosynthetic downregulation. *Physiologia Plantarum* **123**, 348-358.

Armstrong RD, McCosker K, Johnson SB, Walsh KB, Millar G, Kuskopf B, Standley J, Probert ME (1999) Legume and opportunity cropping systems in central Queensland. 1. Legume growth, nitrogen fixation, and water use. *Australian Journal of Agricultural Research* **50**, 909-924.

Athar M, Johnson DA (1997) Effects of drought on the growth and survival of *Rhizobium meliloti* strains from Pakistan and Nepal. *Journal of Arid Environments* **35**, 335-340.

Auricht GC (1999) Lucerne: an Australian breeding success story. In 'Proceedings of the 11th Australian Plant Breeding Conference'. Adelaide, South Australia. (Eds. P Langride, L Malcom). (CRC for Molecular Plant Breeding).

Australian Bureau of Meteorology (2009) 'Climate Data Online ' Available at http://www.bom.gov.au/climate/averages/ Verified 26 February 2010

Avice JC, Lemaire G, Ourry A, Boucaud J (1997a) Effects of the previous shoot removal frequency on subsequent shoot regrowth in two *Medicago sativa* L. cultivars. *Plant and Soil* **188**, 189-198.

Avice JC, Ourry A, Lemaire G, Boucaud J (1996a) Nitrogen and carbon flows estimated by ^{15}N and ^{13}C pulse-chase labelling during regrowth of alfalfa. *Plant Physiology* **112**, 281-290.

Avice JC, Ourry A, Lemaire G, Volenec JJ, Boucaud J (1997b) Root protein and vegetative storage protein are key organic nutrients for alfalfa shoot regrowth. *Crop Science* **37**, 1187-1193.

Avice JC, Ourry A, Volenec JJ, Lemaire G, Boucaud J (1996b) Defoliation-induced changes in abundance and immuno-localization of vegetative storage proteins in taproots of *Medicago sativa*. *Plant Physiology and Biochemistry (Paris)* **34**, 561-570.

Balde AT, Vandersall JH, Erdman RA, Reeves JB, Glenn BP (1993) Effect of stage of maturity of alfalfa and orchardgrass on in situ dry matter and crude protein degradability and amino acid composition. *Animal Feed Science and Technology* **44**, 29-43

Baligar VC, Pitta GVE, Gamma EEG, Schaffert RE, Bahia Filho AF, Clark RB (1997) Soil acidity effects on nutrient use efficiency in exotic maize genotypes. *Plant and Soil* **192**, 9-13.

Barber LD, Joern BC, Volenec JJ, Cunningham SM (1996) Supplemental nitrogen effects on alfalfa regrowth and nitrogen mobilization from roots. *Crop Science* **36**, 1217-1223.

Barnes DK, Bingham ET, Murphy RP, Hunt OJ, Beard DF, Skrdla WH, Teuber LR (1977) Alfalfa germplasm in the United States: Genetic vulnerability, use, improvement, and maintenance. USDA Technical Bulletin 1571.

Barnes DK, Goplen BP, Baylor JE (1988) Highlights in the USA and Canada. In 'Alfalfa and Alfalfa Improvement '. (Eds. AA Hanson, DK Barnes, RR Hill) pp. 1-24. (American Society Agronomy: Madison, WI, USA).

Barrett-Lennard EG, Leighton PD, Buwalda F, Gibbs J, Armstrong W, Thomson CJ, Greenway H (1988) Effects of growing wheat in hypoxic nutrient solutions and of subsequent transfer to aerated solutions. I. Growth and carbohydrate status of shoots and roots. *Australian Journal of Plant Physiology* **15**, 585-598.

Barta AL (1988a) Response of field grown alfalfa to root waterlogging and shoot removal. I. Plant injury and carbohydrate and mineral content of roots. *Agronomy Journal* **80**, 889-892.

Barta AL (1988b) Response of field grown alfalfa to root waterlogging and shoot removal. II. Nitrogen and fermentation metabolism. *Agronomy Journal* **80**, 893-896.

Belanger G, Richards JE, McQueen RE (1992) Effects of harvesting systems on yield, persistence and nutritive value of alfalfa. *Canadian Journal of Plant Science* **72**, 793-799.

Bell CE, Guerrero JN (1997) Sheep grazing effectively controls weeds in seedling alfalfa. *California Agriculture* **51**, 19-23.

Bell LW, Williams AH, Ryan MH, Ewing MA (2007) Water relations and adaptations to increasing water deficit in three perennial legumes, *Medicago sativa*, *Dorycnium hirsutum* and *Dorycnium rectum*. *Plant and Soil* **290**, 231-243.

Bellotti W, Auricht GC, Williams R, Hill MJ (1998) The evolving role of lucerne in Australia. In 'Proceedings of the 36th North American Alfalfa Improvement Conference'. Bozeman, MT, USA. (Ed. J Bouton) p. 62.

Bennett OL, Doss BD (1960) Effect of soil moisture level on root distribution of cool season forage species. *Agronomy Journal* **52**, 204-207.

Berg WK, Cunningham SM, Brouder SM, Joern BC, Johnson KD, Santini J, Volenec JJ (2005) Influence of phosphorus and potassium on lucerne yield and yield components. *Crop Science* **45**, 297-304.

Berg WK, Cunningham SM, Brouder SM, Joern BC, Johnson KD, Santini JB, Volenec JJ (2007) The long-term impact of phosphorus and potassium fertilization on alfalfa yield and yield components. *Crop Science* **47**, 2198-2209.

Berg WK, Cunningham SM, Brouder SM, Joern BC, Johnson KD, Volenec JJ (2009) Influence of phosphorus and potassium on alfalfa yield, taproot C and N pools, and transcript levels of key genes after defoliation. *Crop Science* **49**, 974-982.

Beuselinck PR, Bouton JH, Lamp WO, Matches AG, McCaslin MH, Nelson CJ, Rhodes LH, Volenec JJ (1994) Improving legume persistence in forage crop systems. *Journal of Production Agriculture* **7**, 311-322.

Blair G (1997) Matching pastures to the Australian environment. In 'Pasture Production and Management '. (Eds. JV Lovett, JM Scott) pp. 88-109. (Inkata Press: Port Melbourne).

Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC (1984) A rapid, sensitive method for detection of alkaline-phosphatase conjugated anti-antibody on Western blots. *Analytical Biochemistry* **136**, 175-179

Bolger TP, Meyer DW (1983) Influence of plant density on alfalfa yield and quality. In 'Proceedings if the 1983 Forage and Grasslands Conference' pp. 37-41. (American Forage and Grassland Council: Eau Claire, WI, USA).

Bolton JL (1962) 'Alfalfa: Botany, Cultivation and Utilization.' (Interscience Publishers: New York, NY, USA).

Boschma SP, Williams RW (2008) Using morphological traits to identify persistent lucernes for dryland agriculture in NSW, Australia. *Australian Journal of Agricultural Research* **59**, 69-79.

Bouton JH, Gates RN (2003) Grazing-tolerant alfalfa cultivars perform well under rotational stocking and hay management. *Agronomy Journal* **95**, 1461-1464.

Bouton JH, Smith SR (1998) Standard test to characterize alfalfa cultivar tolerance to intensive grazing with continuous stocking. In 'Standard tests to characterize alfalfa cultivars'. (Ed. CC Fox) pp. A-8. (North American Alfalfa Improvement Conference: Bozeman, MT, USA).

Bowman AM, Peoples MB, Smith W, Brockwell J (2002) Factors affecting nitrogen fixation by dryland lucerne in central-western New South Wales. *Australian Journal of Experimental Agriculture* **42**, 439-451.

Boyce PJ, Volenec JJ (1992) Taproot carbohydrate concentrations and stress tolerance of contrasting alfalfa genotypes. *Crop Science* **32**, 757-761.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Brouwer DJ, Duke SH, Osborn TC (2000) Mapping genetic factors associated with winter hardiness, fall growth, and freezing injury in autotetraploid alfalfa. *Crop Science* **40**, 1387-1396.

Brown AJ (1993) A review of soil sampling for chemical analysis. *Australian Journal of Experimental Agriculture* **33**, 983-1006.

Brown HE, MootDJ (2003) Leaf appearance in seedling lucerne crops. In 'Solutions for a better environment: Proceedings of the 11th Australian Agronomy Conference,'. Geelong, Victoria pp. 79-79. (Australian Society of Agronomy).

Brown HE, Moot DJ, Fletcher AL, Jamieson PD (2009) A framework for quantifying water extraction and water stress responses of perennial lucerne. *Crop and Pasture Science* **60**, 785-794.

Brown HE, Moot DJ, Pollock KM (2005a) Herbage production, persistence, nutritive characteristics and water use of perennial forages grown over 6 years on a Wakanui silt loam. *New Zealand Journal of Agricultural Research* **48**, 423-439.

Brown HE, Moot DJ, Teixeira EI (2005b) The components of lucerne (*Medicago sativa*) leaf area index respond to temperature and photoperiod in a temperate environment. *European Journal of Agronomy* **23**, 348-358.

Brown HE, Moot DJ, Teixeira EI (2006) Radiation use efficiency and biomass partitioning of lucerne (*Medicago sativa*) in a temperate climate. *European Journal of Agronomy* **25**, 319-327.

Brown PW, Tanner CB (1983) Alfalfa stem and leaf growth during water stress. *Agronomy Journal* **75**, 799-805.

Brummer EC, Bouton JH (1991) Plant traits associated with grazing-tolerant alfalfa. *Agronomy Journal* **83**, 996-1000.

Brummer EC, Shah MM, Luth D (2000) Reexamining the relationship between fall dormancy and winter hardiness in alfalfa. *Crop Science* **40**, 971-977.

Bryant AM (1978) Milk yield and composition from cows grazing lucerne. *Proceedings of the New Zealand Society of Animal Production* **38**, 185-190.

Bryant HT, Blaser RE, Hammes RCJ, Hardison WA (1961) Comparison of continuous and rotational grazing of three forage mixtures by dairy cows. *Journal of Dairy Science* **44**, 1742-1750.

Bula RJ, Smith D, Hodgson HJ (1965) Cold resistance in alfalfa at two diverse latitudes. *Agronomy Journal* **48**, 153-156.

Butler JHA (1988) Growth and N_2 fixation by field grown *Medicago littoralis* in response to added nitrate and competition from *Lolium multiflorum*. *Soil Biology & Biochemistry* **20**, 863-868.

Buxton DR, Hornstein JS, Wedin WF (1985) Forage quality in stratified canopies of alfalfa, birdsfoot trefoil and red clover. *Crop Science* **25**, 273-279.

Cameron DG (1973) Lucerne in wet soils - the effect of stage of regrowth, cultivar, air temperature, and root temperature. *Australian Journal of Agricultural Research* **24**, 851-861.

Campling RC (1984) Lucerne, red clover, and other forage legumes: Feeding value and animal production In 'Forage legumes. Occasional symposium No. 16'. (Ed. DJ Thomson) pp. 140 - 146. (British Grassland Society: Hurley, UK).

Carter MR, Richards JE (2000) Soil and alfalfa response after amelioration of subsoil acidity in a fine sandy loam Podzol in Prince Edward Island. *Canadian Journal of Soil Science* **80**, 607-615.

Carter PR, Sheaffer CC (1983a) Alfalfa response to soil water deficits. I. Growth, forage quality, yield, water use, and water-use efficiency. *Crop Science* **23**, 669-675.

Carter PR, Sheaffer CC (1983b) Alfalfa response to soil water deficits. II. Plant water potential, leaf conductance, and canopy temperature relationships. *Crop Science* **23**, 676-680.

Carter PR, Sheaffer CC, Voorhees WB (1982) Root growth, herbage yield, and plant water status of alfalfa cultivars. *Crop Science* **22**, 425-427.

Castonguay Y, Laberge S, Brummer EC, Volenec JJ (2006) Alfalfa winter hardiness: a research retrospective and integrated perspective. *Advances in Agronomy* **90**, 203-265.

Castonguay Y, Nadeau P, Lechasseur P, Chouinard L (1995) Differential accumulation of carbohydrates in alfalfa cultivars of contrasting winter hardiness. *Crop Science* **35**, 509-516.

Ceccarelli S, Erskine W, Hamblin J, Grando S (1994) Genotype by environment interaction and international breeding programmes. *Experimental Agriculture* **30**, 177-187.

Ceccarelli S (1996) Positive interpretation of genotype by environment interactions in relation to sustainability and biodiversity. In 'Plant adaptation and crop improvement.'. (Eds M Cooper, GL Hammer) pp. 467-486. (CAB International: Wallingford).

Chocarro C, Lloveras J, Fanlo R (2005) Effects of winter grazing on spring production of lucerne under Mediterranean conditions. *Grass and Forage Science* **60**, 146-150.

Clarke RTJ, Reid CSW (1974) Foamy bloat of cattle. A review. *Journal of Dairy Science* **57**, 753-785.

Close TJ (1996) Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum* **97**, 795-803.

Close TJ (1997) Dehydrins: a commonality in the response of plants to dehydration and low temperature. *Physiologia Plantarum* **100**, 291-296.

Cohen Y, Bieloria H, Dovrat A (1972) Effect of timing of irrigation on total nonstructural carbohydrate level in roots and on seed yield of alfalfa (*Medicago sativa* L.) *Crop Science* **12**, 634-636.

Collino DJ, Dardanelli JL, De Luca MJ, Racca RW (2005) Temperature and water availability effects on radiation and water use efficiencies in alfalfa

(*Medicago sativa* L.). *Australian Journal of Experimental Agriculture* **45**, 383-390.

Collins M, Duke SH (1981) Influence of potassium-fertilization rate and form on photosynthesis and N_2 fixation of alfalfa. *Crop Science* **21**, 481-485.

Collins M, Taylor TH (1980) Yield and quality of alfalfa harvested during autumn and winter and harvest effects on the spring crop. *Agronomy Journal* **72**, 839-844.

Commonwealth of Australia (2002) 'Australian Natural Resources Atlas' Available at http://www.anra.gov.au/topics/agriculture/statistics/stats/australia/lucerne-pure-year-ending-31-march-ha.html Verified 26 February 2010

Conrad HR, Van Keuren RW, Dehority BA (1982) Top-grazing high-protein forages with lactating cows. In 'Proceedings of the forteenth international grasslands congress'. Lexington, KY, USA pp. 690-692.

Cooper M, Eeuwijk Fv, Chapman SC, Podlich DW, Loffler C (2006) Genotype-by-environment interactions under water-limited conditions. In 'Drought adaptation in cereals'. (Ed. JM Ribaut) pp. 51-96. (Haworth Press: Binghamton, NY).

Cowett ER, Sprague MA (1962) Factors affecting tillering in alfalfa. *Agronomy Journal* **54**, 294-297.

Cowett ER, Sprague MA (1963) Effect of stand density and light intensity on the microenvironment and stem production of alfalfa. *Agronomy Journal* **55**, 432-434.

Cunningham SM, Gana JA, Volenec JJ, Teuber LR (2001) Winter hardiness, root physiology, and gene expression in successive fall dormancy selections from 'Mesilla' and 'CUF 101' alfalfa. *Crop Science* **41**, 1091-1098.

Cunningham SM, Nadeau P, Castonguay Y, Laberge S, Volenec JJ (2003) Raffinose and stachyose accumulation, galactinol synthase expression, and winter injury of contrasting alfalfa germplasms. *Crop Science* **43**, 562-570.

Cunningham SM, Volenec JJ (1996) Purification and characterization of vegetative storage proteins from alfalfa (*Medicago sativa* L.) taproots. *Journal of Plant Physiology* **147**, 625-632.

Cunningham SM, Volenec JJ (1998) Seasonal carbohydrate and nitrogen metabolism in roots of contrasting alfalfa (*Medicago sativa* L.) cultivars. *Journal of Plant Physiology* **153**, 220-225.

Cunningham SM, Volenec JJ, Teuber LR (1998) Plant survival and root and bud composition of alfalfa populations selected for contrasting fall dormancy. *Crop Science* **38**, 962-969.

Daday H, Mottershead BE, Rogers VE (1961) Performance and interactions in varieties of lucerne (*Medicago sativa* L.). *Australian Journal of Experimental Agriculture and Animal Husbandry* **1**, 67-72.

Davies PJ (1987) The plant hormones: Their nature, occurrence, and functions. In 'Plant Hormones and Their Role in Plant Growth and Development'. (Ed. PJ Davies) pp. 1-11. (Kluwer Academic Publishers: Boston, MA, USA).

Dear BS, Peoples MB, Cocks PS, Swan AD, Smith AB (1999) Nitrogen fixation by subterranean clover (*Trifolium subterraneum* L.) growing in pure culture and in mixtures with varying densities of lucerne (*Medicago sativa* L.) or phalaris

(*Phalaris aquatica* L.). *Australian Journal of Agricultural Research* **50**, 1047-1058.

Dear BS, Peoples MB, Hayes RC, Swan AD, Chan KY, Oates AA, Morris SG, Orchard BA (2010) Effect of gypsum on establishment, persistence and productivity of lucerne and annual pasture legumes on two grey Vertosols in southern New South Wales. *Crop & Pasture Science* **61**, 435-449.

Dhont C, Castonguay Y, Avice JC, Chalifour FP (2006a) VSP accumulation and cold-inducible gene expression during autumn hardening and overwintering of alfalfa. *Journal of Experimental Botany* **57**, 2325-2337.

Dhont C, Castonguay Y, Nadeau P, Belanger G, Chalifour FP (2002) Alfalfa root carbohydrates and regrowth potential in response to fall harvests. *Crop Science* **42**, 754-765.

Dhont C, Castonguay Y, Nadeau P, Belanger G, Chalifour FP (2003) Alfalfa root nitrogen reserves and regrowth potential in response to fall harvests. *Crop Science* **43**, 181-194.

Dhont C, Castonguay Y, Nadeau P, Belanger G, Drapeau R, Chalifour FP (2004) Untimely fall harvest affects dry matter yield and root organic reserves in field-grown alfalfa. *Crop Science* **44**, 144-157.

Dhont C, Castonguay Y, Nadeau P, Belanger G, Drapeau R, Laberge S, Avice JC, Chalifour FP (2006b) Nitrogen reserves, spring regrowth and winter survival of field-grown alfalfa (*Medicago sativa*) defoliated in the autumn. *Annals of Botany* **97**, 109-120.

Digby PGN (1979) Modified joint regression analysis for incomplete variety X environment data. *Journal of Agricultural Science, UK* **93**, 81-86.

Dillon P, Roche JR, Shalloo L, Horan B (2005) Optimising financial return from grazing in temperate pastures. In 'Utilisation of grazed grass in temperate animal systems. Proceedings of a satellite workshop of the XX International Grassland Congress. July 2005'. (Ed. JJ Murphy) pp. 131 - 147. (Wageningen Academic Publishing: Cork, Ireland).

Doonan B (2003) People, price, property, production - What really drives profit? In 'The 2003 IMPACT Dairy Business of the Year Award'. Smithton, Tasmainia pp. 10-11. (Department of Primary Industries Water and Enviroment, Tasmania).

Doorenbos J, Pruitt WO (1977) Guidelines for predicting crop water requirements. In 'FAO Irrigation and Drainage Paper no. 24' (Food and Agricultural Organisation of the United Nations: Rome, Italy)

Dougherty CT, Collins M, Bradley NW, Lauriault LM, Cornelius PL (1992) The effects of poloxalene on ingestion by cattle grazing lucerne. *Grass and Forage Science* **47**, 180-188.

Douglas JA (1986) The production and utilization of lucerne in New Zealand. *Grass and Forage Science* **41**, 81-128.

Durand JL, Lemaire G, Gosse G, Chartier M (1989) Analyse de la conversion de l'energie solaire en matiere seche par un peuplement de lucerne (*Medicago sativa* L.) soumis a un deficit hydrique. *Agronomie* **9**, 599-607.

Erice G, Irigoyen JJ, Perez P, Martinez-Carrasco R, Sanchez-Diaz M (2006) Effect of elevated CO_2, temperature and drought on photosynthesis of nodulated alfalfa during a cutting regrowth cycle. *Physiologia Plantarum* **126**, 458-468.

Erice G, Irigoyen JJ, Sanchez-Diaz M, Avice JC, Ourry A (2007) Effect of drought, elevated CO_2 and temperature on accumulation of N and vegetative storage

proteins (VSP) in taproot of nodulated alfalfa before and after cutting. *Plant Science* **172**, 903-912.

Fankhauser JJ, Volenec JJ (1989) Root vs. shoot effects on herbage regrowth and carbohydrate metabolism of alfalfa. *Crop Science* **29**, 735-740.

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6-13.

Ferguson JE, Murphy RP (1973) Comparison of trifoliolate and multifoliolate phenotypes of alfalfa (*Medicago sativa* L.). *Crop Science* **13**, 463-465.

Fick GW, Holt DA, Lugg DG (1988) Environmental Physiology and Crop Growth. In 'Alfalfa and Alfalfa Improvement'. (Eds. AA Hanson, DK Barnes, RR Hill) pp. 163-191. (American Society of Agronomy: Madison, WI, USA).

Finlay KW, Wilkinson GN (1963) The analysis of adaptation in a plant-breeding programme. *Australian Journal of Agricultural Research* **14**, 742-754.

Foy CD (1984) Physiological effects of hydrogen, aluminium and manganese toxicities in acid soils. In 'Soil Acidity and Liming'. (Ed. F Adams) pp. 57-97 (American Society of Agronomy: Madison, WI, USA).

Frame J, Charlton JFL, Laidlaw AS (1998) Lucerne (syn. Alfalfa). In 'Temperate Forage Legumes' pp. 107-179. (CAB International: New York, NY, USA).

Frame J, Laidlaw S (2005) Prospects for temperate forage legumes. In 'Grasslands: Developments, Opportunities, Perspectives'. (Eds. SG Reynolds, J Frame) pp. 1-28. (Science Publishers in association with the Food and Agriculture Organization of the United Nations: Enfield, NH, USA).

Fulkerson B, Doyle P (2001) 'The Australian Dairy Industry.' (Victorian Department of Natural Resources and Environment: Melbourne, Victoria).

Gana JA, Kalengamaliro NE, Cunningham SM, Volenec JJ (1998) Expression of β-amylase from alfalfa taproots. *Plant Physiology* **118**, 1495-1505.

Gibbs J, Greenway H (2003) Mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. *Functional Plant Biology* **30**, 1-47.

Goh KM, Mansur I, Mead DJ, Sweet GB (1996) Biological nitrogen fixing capacity and biomass production of different understorey pastures in a *Pinus radiata*-pasture agroforestry system in New Zealand. *Agroforestry Systems* **34**, 33-49.

Gosse G, Lemaire G, Chartier M, Balfourier F (1988) Structure of a lucerne population (*Medicago sativa* L.) and dynamics of stem competition for light during regrowth. *Journal of Applied Ecology* **25**, 609-617.

Gossen BD, Horton PR, Wright SBM, Duncan CH (1994) Field response of alfalfa to harvest frequency, cultivar, crown pathogens, and soil fertility: I. Survival and yield. *Agronomy Journal* **86**, 82-88.

Graber LF, Nelson NT, Luekel WA, Albert WB (1927) Organic food reserves in relation to the growth of alfalfa and other perennial herbaceous plants. Bulletin of the Wisconsin Agricultural Experiment Station No. 80.

Graham PH (1992) Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. *Canadian Journal of Microbiology* **38**, 475-484.

Grainger C, Fulkerson W, Cowan T, Michell P (1991) Grazing Management and Utilisation of Temperate and Tropical Pastures. In 'Feedbase 2000: a workshop to determine the priorities for research into soils, pastures and

fodder crops in Australian dairy production systems'. (Eds. B Bartsch, W Mason) pp. 59-69. (CSIRO Publications: East Melbourne, Victoria).

Gramshaw D (1981) Factors affecting lucerne production. *Queensland Agricultural Journal* **107**, 25-28.

Gramshaw D, Lloyd DL, Lowe KF (1981) Growing new lucernes in Queensland. *Queensland Agricultural Journal* **107**, 249-254.

Gramshaw D, Lowe KF, Lloyd DL (1993) Effect of cutting interval and winter dormancy on yield, persistence, nitrogen concentration, and root reserves of irrigated lucerne in the Queensland subtropics. *Australian Journal of Experimental Agriculture* **33**, 847-854.

Grandfield CO (1943) Food reserves and their translocation to the crown buds as related to cold and drought resistance in alfalfa. *Journal of Agricultural Research* **67**, 33-47.

Grewal HS, Williams R (2003) Liming and cultivars affect root growth, nodulation, leaf to stem ratio, herbage yield, and elemental composition of alfalfa on an acid soil. *Journal of Plant Nutrition* **26**, 1683-1696.

Grimes DW, Wiley PL, Sheesley WR (1992) Alfalfa yield and plant water relations with variable irrigation. *Crop Science* **32**, 1381-1387.

Haagenson DM, Cunningham SM, Joern BC, Volenec JJ (2003a) Autumn defoliation effects on alfalfa winter survival, root physiology, and gene expression. *Crop Science* **43**, 1340-1348.

Haagenson DM, Cunningham SM, Volenec JJ (2003b) Root physiology of less fall dormant, winter hardy alfalfa selections. *Crop Science* **43**, 1441-1447.

Habben JE, Volenec JJ (1990) Starch grain distribution in taproots of defoliated *Medicago sativa* L. *Plant Physiology* **94**, 1056-1061.

Habben JE, Volenec JJ (1991) Amylolytic activity in taproots of diploid and tetraploid *Medicago sativa* L. *Annals of Botany* **68**, 393-400.

Halim RA, Buxton DR, Hattendorf MJ, Carlson RE (1989a) Water-deficit effects on alfalfa at various growth stages. *Agronomy Journal* **81**, 765-770.

Halim RA, Buxton DR, Hattendorf MJ, Carlson RE (1989b) Water-stress effects on alfalfa forage quality after adjustment for maturity differences. *Agronomy Journal* **81**, 189-194.

Hall RH (1973) Cytokinins as a probe of developmental processes. *Annual Review of Plant Physiology* **24**, 415-444.

Halloran GM and Luckett DJ (1993) Crop improvement. In 'Principles of field crop production 3rd edition. (Eds J Pratley) pp. 118-199 (Oxford University Press: Melbourne)

Hattendorf MJ, Evans DW, Peaden RN (1990) Canopy temperature and stomatal conductance of water-stressed dormant and nondormant alfalfa types. *Agronomy Journal* **82**, 873-877.

Heichel GH, Delaney RH, Cralle HT (1988) Carbon Assimilation, Partitioning and Utilization. In 'Alfalfa and Alfalfa Improvement'. (Eds. AA Hanson, DK Barnes, RR Hill) pp. 195-228. (American Society of Agronomy: Madison, WI, USA).

Heichel GH, Henjum KI (1990) Fall dormancy response of alfalfa investigated with reciprocal cleft grafts. *Crop Science* **30**, 1123-1127.

Heichel GH, Henjum KI (1991) Dinitrogen fixation, nitrogen transfer, and productivity of forage legume-grass communities. *Crop Science* **31**, 202-208.

Heinrichs DH (1973) Winterhardiness of alfalfa cultivars in southern Saskatchewan. *Canadian Journal of Plant Science* **53**, 773-777.

Heinze Walz GmbH (1999) 'Photosynthesis yield analyzer mini-PAM. Portable Chlorophyll Fluorometer. Handbook of Operation.' (Heinz Walz GmbH: Eichenring, Germany).

Helyar KR, Anderson AJ (1970) Responses of five pasture species to phosphorus, lime, and nitrogen on an infertile acid soil with a high phosphate sorption capacity. *Australian Journal of Agricultural Research* **21**, 677-692.

Hendershot KL, Volenec JJ (1989) Shoot growth, dark respiration, and nonstructural carbohydrates of contrasting alfalfa genotypes. *Crop Science* **29**, 1271-1275.

Hendershot KL, Volenec JJ (1992) Taproot nitrogen accumulation and use in overwintering alfalfa (*Medicago sativa* L.). *Journal of Plant Physiology* **141**, 68-74.

Hendershot KL, Volenec JJ (1993) Nitrogen pools in taproots of *Medicago sativa* L. after defoliation. *Journal of Plant Physiology* **141**, 129-135.

Hendrickson JR, Liebig MA, Berdahl JD (2008) Responses of *Medicago sativa* and *M. falcata* type alfalfas to different defoliation times and grass competition. *Canadian Journal of Plant Science* **88**, 61-69.

Hendry GW (1923) Alfalfa in history. *Journal of the American Society of Agronomy* **15**, 171-176.

Hill MJ (1996) Potential adaptation zones for temperate pasture species as constrained by climate: a knowledge-based logical modelling approach. *Australian Journal of Agricultural Research* **47**, 1095-1117.

Hodgkinson KC (1969) The utilization of root organic compounds during the regeneration of lucerne. *Australian Journal of Biological Science* **22**, 1113-1123.

Hodgkinson KC (1973) Establishment and growth of shoots following low and high cutting of lucerne in relation to the pattern of nutrient uptake. *Australian Journal of Agricultural Research* **24**, 497-510.

Hodgkinson KC (1974) Influence of partial defoliation on photosynthesis, photorespiration and transpiration by lucerne leaves of different ages. *Australian Journal of Plant Physiology* **1**, 561-578.

Hodgson HJ (1964) Effect of photoperiod on development of cold resistance in alfalfa. *Crop Science* **4**, 302-330.

Howieson JG, Ewing MA (1986) Acid tolerance in the *Rhizobium meliloti* – *Medicago symbiosis*. *Australian Journal of Agricultural Research* **37**, 55-64.

Humphries AW, Auricht GC (2001) Breeding lucerne for Australia's southern dryland cropping environments. *Australian Journal of Agricultural Research* **52**, 153-169.

Humphries AW, Ballard RA, Garnett TP, Charman N, Zhang XG, Auricht G (2006a) An integrated approach to breeding acid aluminium tolerant lucerne. In 'Proceedings of the 40th North American Alfalfa Improvement Conference and 19th Trifolium Conference'. Bloomington, MN, USA. (The North American Alfalfa Improvement Conference).

Humphries AW, Hughes SJ (2006) Preliminary evaluation of diverse lucerne (*Medicago sativa* sspp.) germplasm to identify new material for livestock and cropping based Australian farming systems. *Australian Journal of Agricultural Research* **57**, 1297-1306.

Humphries AW, Kobelt ET, Bellotti WD, Auricht GC (2006b) Tolerance of Australian lucerne (*Medicago sativa*) germplasm to grazing by sheep. *Australian Journal of Experimental Agriculture* **46**, 1263-1270

Irigoyen JJ, Emerich DW, Sanchez-Diaz M (1992a) Alfalfa leaf senescence induced by drought stress: photosynthesis, hydrogen peroxide metabolism, lipid peroxidation and ethylene evolution. *Physiologia Plantarum* **84**, 67-72.

Irigoyen JJ, Sanchez-Diaz M, Emerich DW (1992b) Transient increase of anaerobically-induced enzymes during short-term drought of alfalfa root nodules. *Journal of Plant Physiology* **139**, 397-402.

Irwin JAG, Lloyd DL, Lowe KF (2001) Lucerne biology and genetic improvement - an analysis of past activities and future goals in Australia. *Australian Journal of Agricultural Research* **52**, 699-712.

Isbell RF (2002) 'The Australian soil classification ' (CSIRO Publishing: Collingwood).

Ivashuta S, Uchiyama K, Gau M, Shimamoto Y (2002) Linear amplification coupled with controlled extension as a means of probe amplification in a cDNA array and gene expression analysis during cold acclimation in alfalfa (*Medicago sativa* L.). *Journal of Experimental Botany* **53**, 351-359.

Janson CG (1975) Interaction of maturity stage and defoliation duration in the simulated grazing of irrigated lucerne. *New Zealand Journal of Experimental Agriculture* **3**, 63-69.

Jeffrey SJ, Carter JO, Moodie KM, Beswick AR (2001) Using spatial interpolation to construct a comprehensive archive of Australian climate data. *Environmental Modelling and Software* **16**, 309-330

Jennings JA, Nelson CJ (1998) Influence of soil texture on alfalfa autotoxicity. *Agronomy Journal* **90**, 54-58.

Jennings JA, Nelson CJ (2002) Zone of autotoxic influence around established alfalfa plants. *Agronomy Journal* **94**, 1104-1111.

Jones ER, Carroll RB, Swain RH, Bell KW (1984) Evaluation of factors affecting yield reduction in mature alfalfa (*Medicago sativa* L.). *Canadian Journal of Plant Science* **64**, 901-907.

Joost RE, Hoveland CS (1986) Root development of sericea lespedeza and alfalfa in acid soils. *Agronomy Journal* **78**, 711-714.

Juan NA, Sheaffer CC, Barnes DK (1994) Root and crown characteristics of alfalfas varying in fall dormancy. *Canadian Journal of Plant Science* **74**, 125-127.

Julier B, Huyghe C, Ecalle C (2000) Within- and among-cultivar genetic variation in alfalfa: forage quality, morphology, and yield. *Crop Science* **40**, 365-369.

Justes E, Thiebeau P, Avice JC, Lemaire G, Volenec JJ, Ourry A (2002) Influence of summer sowing dates, N fertilization and irrigation on autumn VSP accumulation and dynamics of spring regrowth in alfalfa (*Medicago sativa* L.). *Journal of Experimental Botany* **53**, 111-121.

Kaehne I, Kobelt ET, Horsnell JA (1993) Contribution of undomesticated germplasm to development of persistent, grazing tolerant lucerne. In 'Proceedings of the

17th International Grassland Congress'. Palmerston North, New Zealand. (Ed. MJ Baker) pp. 215-216. (New Zealand Grassland Association).

Kaehne ID, Lake A (1982) Lucerne Varieties - 1982. (South Australian Department of Agriculture Adelaide, South Australia).

Kalengamaliro NE, Volenec JJ, Cunningham SM, Joern BC (1997) Seedling development and deposition of starch and storage proteins in alfalfa roots. *Crop Science* **37**, 1194-1200.

Kalu BA, Fick GW (1981) Quantifying morphological development of alfalfa for studies of herbage quality. *Crop Science* **21**, 267-271.

Kanneganti VR, Bland WL, Undersander DJ (1998a) Modeling freezing injury in alfalfa to calculate forage yield: II. Model validation and example simulations. *Agronomy Journal* **90**, 698-704.

Kanneganti VR, Rotz CA, Walgenbach RP (1998b) Modeling freezing injury in alfalfa to calculate forage yield: I. Model development and sensitivity analysis. *Agronomy Journal* **90**, 687-697.

Karamanos AJ, Papastylianou PT, Stavrou J, Avgoulas C (2009) Effects of water shortage and air temperature on seed yield and seed performance of lucerne (*Medicago sativa* L.) in a Mediterranean environment. *Journal of Agronomy and Crop Science* **195**, 408-419.

Kelly K, Chopping G, Prance T, Crocos A (1991) Annual Pastures. In 'Feedbase 2000: a workshop to determine the priorities for research into soils, pastures and fodder crops in Australian dairy production systems'. (Eds. B Bartsch, W Mason) pp. 20-31. (Dairy Research and Development Corporation: East Melbourne, Victoria).

Kemp DR, Michalk DL (2005) Australian temperate grasslands: changing philosophies and future prospects. In 'Grasslands: Developments, Opportunities, Perspectives'. (Eds. SG Reynolds, J Frame). (Science Publishers in association with Food and Agriculture Organization of the United Nations: Enfield, NH, USA).

Kemp PD, Matthew C, Lucas RJ (1999) Pasture Species and Cultivars. In 'New Zealand Pasture and Crop Science'. (Eds. JGH White, HJ Hodgson) pp. 83-99. (Oxford University Press: Auckland, NZ).

Kendall WA, Shaffer JA, Hill RRJ (1994) Effect of temperature and water variables on the juvenile growth of lucerne and red clover. *Grass and Forage Science* **49**, 264 - 269.

Khaiti M, Lemaire G (1992) Dynamics of shoot and root growth of lucerne after seeding and after cutting. *European Journal of Agronomy* **1**, 241-247.

Khu DM, Reyno R, Brummer EC, Bouton JH, Monteros MJ (2009) Identification and QTL mapping of aluminium tolerance in tetraploid alfalfa. In 'Proceedings of the American Society of Agronomy, Crop Science Society of America and Soil Science Society of America 2009 International Joint Annual Meetings'. Pittsburgh, PA USA.

Kibbe WA (2007) OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research* **35**, W43-W46.

Kim TH, Ourry A, Boucard J, Lemaire G (1993) Partitioning of nitrogen derived from N_2 fixation and reserves in nodulated *Medicago sativa* L. during regrowth. *Journal of Experimental Botany* **44**, 555-562.

Kitchen NR, Buchholz DD, Nelson CJ (1990) Potassium fertilizer and potato leafhopper effects on alfalfa growth. *Agronomy Journal* **82**, 1069-1074.

Klebesadel LJ (1971) Selective modification of alfalfa toward acclimatization in a subarctic area of severe winter stress. *Crop Science* **11**, 609-614.

Knox J, Campbell S, Field B, Thompson R, Hall E (2006) 'Species for Profit: A Guide for Tasmanian Pastures and Field Crops.' (Tasmanian Department of Primary Industries, Water and Environment: Kings Meadows, Tasmania).

Kohl RA, Kolar JJ (1976) Soil water uptake by alfalfa. *Agronomy Journal* **68**, 536-538.

Ku SB, Edwards GE (1977) Oxygen inhibition of photosynthesis. 1. Temperature-dependence and relation to O_2-CO_2 solubility ratio. *Plant Physiology* **59**, 986-990.

Ku SB, Hunt LA (1973) Effects of temperature on the morphology and photosynthetic activity of newly matured leaves of alfalfa. *Canadian Journal of Botany* **51**, 1907-1916.

Ku SB, Hunt LA (1977) Effects of temperature on photosynthesis - irradiance response curves of newly matured leaves of alfalfa. *Canadian Journal of Botany-Revue Canadienne De Botanique* **55**, 872-879.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lamb JFS, Barnes DK, Russelle MP, Vance CP, Heichel GH, Henjum KL (1995) Ineffective and effective nodulated alfalfas demonstrate biological nitrogen fixation continues with high nitrogen fertilization. *Crop Science* **35**, 153 - 157.

Lamb JFS, Sheaffer CC, Rhodes LH, Sulc RM, Undersander DJ, Brummer EC (2006) Five decades of alfalfa cultivar improvement: Impact on forage yield, persistence, and nutritive value. *Crop Science* **46**, 902-909.

Larionov A, Krause A, Miller W (2005) A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics* **6**, 62.

Lazaridou M, Noitsakis V (2005) The effect of cutting on water balance of alfalfa plants. *Journal of Biological Research* **4**, 189-195.

Leach GJ (1968) The growth of the lucerne plant after cutting: I. The effects of cutting at different stages of maturity and at different intensities. *Australian Journal of Agricultural Research* **19**, 517-530.

Leach GJ (1969) Shoot numbers, shoot size, and yield of regrowth in three lucerne cultivars. *Australian Journal of Agricultural Research* **20**, 425-434.

Leach GJ (1970a) An evaluation of lucerne lines at the Waite Agricultural Research Institute, South Australia. *Australian Journal of Experimental Agriculture and Animal Husbandry* **10**, 53-61

Leach GJ (1970b) Shoot growth on lucerne plants cut at different heights. *Australian Journal of Agricultural Research* **21**, 583-591.

Leach GJ (1971) The relation between lucerne shoot growth and temperature. *Australian Journal of Agricultural Research* **22**, 49-59.

Leach GJ (1979) Regrowth characteristics of lucerne under different systems of grazing management. *Australian Journal of Agricultural Research* **30**, 445-465.

Lehane L (1982) Biological control of lucerne aphids. *Rural Press*, 4-10.

Lemaire G, Khaity M, Onnillon B, Allirand JM, Chartier M, Gosse G (1992) Dynamics of accumulation and partitioning of N in leaves, stems and roots of lucerne (*Medicago sativa* L.) in a dense canopy. *Annals of Botany* **70**, 429-435.

Lenssen AW, Sorensen EL, Posler GL, Blodgett SL (1991) Depression of forage quality of alfalfa leaves and stems by *Acyrthosiphon kondoi* (Homoptera: Aphididae). *Environmental Entomology* **20**, 71-76.

Lewis D, Temple-Smith M, Nobel C, Ellington A (1991) Soils. In 'Feedbase 2000: a workshop to determine the priorities for research into soils, pastures and fodder crops in Australian dairy production systems'. (Eds. B Bartsch, W Mason) pp. 13-19. (Dairy Research and Development Corporation: East Melbourne, Victoria).

Li R, Volenec JJ, Joern BC, Cunningham SM (1996) Seasonal changes in nonstructural carbohydrates, protein, and macronutrients in roots of alfalfa, red clover, sweetclover, and birdsfoot trefoil. *Crop Science* **36**, 617-623.

Li R, Volenec JJ, Joern BC, Cunningham SM (1997) Potassium and nitrogen effects on carbohydrate and protein metabolism in alfalfa roots. *Journal of Plant Nutrition* **20**, 511-529.

Li R, Volenec JJ, Joern BC, Cunningham SM (1998) Effects of phosphorus nutrition on carbohydrate and protein metabolism in alfalfa roots. *Journal of Plant Nutrition* **21**, 459-474.

Lissbrant S, Brouder SM, Cunningham SM, Volenec JJ (2010) Identification of fertility regimes that enhance long-term productivity of alfalfa using cluster analysis. *Agronomy Journal* **102**, 580-591.

Lissbrant S, Stratton S, Cunningham SM, Brouder SM, Volenec JJ (2009) Impact of long-term phosphorus and potassium fertilization on alfalfa nutritive value-yield relationships. *Crop Science* **49**, 1116-1124.

Lloveras J, Ferran J, Boixadera J, Bonet J (2001) Potassium fertilization effects on alfalfa in a Mediterranean climate. *Agronomy Journal* **93**, 139-143.

Lloyd DL, Bullen KS, Lowe KF, Irwin JAG (2002) Selecting the right lucerne. In 'The Lucerne Management Handbook '. (Ed. K Bullen) pp. 12-15. (Queensland Department of Primary Industries: Brisbane, Queensland).

Lloyd DL, Gramshaw D, Hilder TB, Ludke DH, Turner JW (1985) Performance of North American and Australian lucernes in the Queensland subtropics. 3. Yield, plant survival and aphid populations in raingrown stands. *Australian Journal of Experimental Agriculture* **25**, 91-99.

Lodge GM (1985) Effects of grazing and haycutting on the yield and persistence of dryland aphid-resistant lucerne cultivars at Tamworth, New South Wales. *Australian Journal of Experimental Agriculture* **25**, 138-148.

Lodge GM (1986) Yield and persistence of irrigated lucernes cut at different frequencies, at Tamworth, New South Wales. *Australian Journal of Experimental Agriculture* **26**, 165-172.

Lodge GM (1991) Management practices and other factors contributing to the decline in persistence of grazed lucerne in temperate Australia: a review. *Australian Journal of Experimental Agriculture* **31**, 713-724.

Lodge GM, Brennan MA, Harden S, Boschma SP (2010) Changes in soil water content under annual, perennial, and shrub-based pastures in an intermittently dry, summer-rainfall environment. *Crop & Pasture Science* **61**, 331-342.

Lodge GM, Gleeson AC (1984) A comparison of methods of estimating lucerne population for monitoring persistence. *Australian Journal of Experimental Agriculture and Animal Husbandry* **24**, 174-177.

Lowe KF, Bowdler TM, Casey ND, Lowe SA, White JA, Pepper PM (2007) Evaluating temperate species for the subtropics. 1 Annual ryegrasses. *Tropical Grasslands* **41**, 9-25.

Lowe KF, Bowdler TM, Casey ND, Lowe SA, White JA, Pepper PM (2008) Evaluating temperate species in the subtropics 2. Perennial grasses. *Tropical Grasslands* **42**, 1-26.

Lowe KF, Gramshaw D, Bowdler TM, Clem RL, Collyer BG (1987) Yield, persistence and field disease assessment of lucerne cultivars and lines under irrigation in the Queensland subtropics. *Tropical Grasslands* **21**, 168-181.

Lowe KF, Gramshaw D, Bowdler TM, Ludke DH (1985) Performance of North American and Australian lucernes in the Queensland subtropics 2. Yield and plant survival in irrigated stands. *Australian Journal of Experimental Agriculture* **25**, 82-90.

Lowe KF, Lloyd DL, Bullen KS (2002) Understanding more about lucerne. In 'The Lucerne Management Handbook'. (Ed. KS Bullen) pp. 1-11. (Queensland Department of Primary Industries: Brisbane, Queensland).

Lutwick LE, Smith AD (1977) Yield and composition of alfalfa and crested wheatgrass, grown singly and in mixture, as affected by N and P fertilizers. *Canadian Journal of Plant Science* **57**, 1027-1083.

Luxmoore RJ, Stolzy LH (1969) Root porosity and growth responses of rice and maize to oxygen supply. *Agronomy Journal* **61**, 202-204.

Ma Y, Li H, Dong Z, Ren G, Dong L (2009) Photosynthesis and transpiration characters of alfalfa (*Medicago sativa*) and their relationship with relevant factors during branching stage. *Animal Husbandry and Feed Science* **1**, 32-35.

MacAdam JW, Nelson CJ (2003) Physiology of Forage Plants. In 'Forages: An Introduction to Grasslands Agriculture'. (Eds. RF Barnes, CJ Nelson, M Collins, KJ Moore) pp. 73-97. (Blackwell Publishing: Ames, IA, USA).

Macleod LB, Jackson LP (1965) Effect of concentration of the aluminium ion on root development and establishment of legume seedlings. *Canadian Journal of Soil Science* **45**, 221 - 234.

Major DJ, Hanna MR, Beasley BW (1991) Photoperiod response characteristics of alfalfa (*Medicago sativa*). *Canadian Journal of Plant Science* **71**, 87-93.

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence - a practical guide. *Journal of Experimental Botany* **51**, 659-668.

McCallum MH, Peoples MB, J. CD (2000) Contributions of nitrogen by field pea (*Pisum sativum* L.) in a continuous cropping sequence compared with a lucerne (*Medicago sativa* L.)-based pasture ley in the Victorian Wimmera. *Australian Journal of Agricultural Research* **51**, 13-22.

McDonald MF (1995) Effects of plant oestrogens in ruminants. *Proceedings of the Nutrition Society of New Zealand* **20**, 43-51.

McDonald P, Edwards RA, Greenhalgh JFD, Morgan CA (2002) 'Animal Nutrition.' (Prentice Hall: London, UK).

McGraw RL, Nelson CJ (2003) Legumes for Northern Areas. In 'Forages, An Introduction to Grasslands Agriculture'. (Eds. RF Barnes, CJ Nelson, M Collins, KJ Moore) pp. 171–190. (Blackwell Publishing Company: Ames, IW, USA).

McIntosh MS, Miller DA (1981) Genetic and soil moisture effects on the branching-root trait in alfalfa. *Crop Science* **21**, 15-18.

McKenzie JS, McLean GE (1980) Changes in the cold hardiness of alfalfa during five consecutive winters at Beaverlodge, Alberta. *Canadian Journal of Plant Science* **60**, 703-712.

McKenzie JS, Pacquin R, Duke SH (1988) Cold and Heat Tolerance. In 'Alfalfa and Alfalfa Improvement'. (Eds. AA Hanson, DK Barnes, RRJ Hill) pp. 259-302. (American Society of Agronomy: Madison, WI, USA).

McKinney GT (1974) Management of lucerne for sheep grazing on the Southern Tablelands of New South Wales. *Australian Journal of Experimental Agriculture and Animal Husbandry* **14**, 726-734.

McLaughlin MJ, Williams CMJ, McKay A, Kirkham R, Gunton J, Jackson KJ, Thompson R, Dowling B, Partington D, Smart MK, Tiller KG (1994) Effect of cultivar on uptake of cadmium by potato tubers. *Australian Journal of Agricultural Research* **45**, 1483-1495.

Melton B, Moutray JB, Bouton JH (1988) Geographic Adaption and Cultivar Selection. In 'Alfalfa and Alfalfa Improvement '. (Eds. AA Hanson, DK Barnes, RRJ Hill) pp. 595-620. (American Society of Agronomy: Madison, WI, USA).

Metochis C, Orphanos PI (1981) Alfalfa yield and water use when forced into dormancy by withholding water during the summer. *Agronomy Journal* **73**, 1048-1050.

Meuriot F, Avice JC, Simon JC, Laine P, Decau ML, Ourry A (2004a) Influence of initial organic N reserves and residual leaf area on growth, N uptake, N partitioning and N storage in alfalfa (*Medicago sativa*) during post-cutting regrowth. *Annals of Botany* **94**, 311-321.

Meuriot F, Noquet C, Avice JC, Volenec JJ, Cunningham SM, Sors TG, Caillot S, Ourry A (2004b) Methyl jasmonate alters N partitioning, N reserves accumulation and induces gene expression of a 32-kDa vegetative storage protein that possesses chitinase activity in *Medicago sativa* taproots. *Physiologia Plantarum* **120**, 113-123.

Michaud R, Lehman WF, Rumbaugh MD (1988) World Distribution and Historical Development. In 'Alfalfa and Alfalfa Improvement'. (Eds. AA Hanson, DK Barnes, RR Hill) pp. 25-91. (American Society of Agronomy: Madison, WI, USA).

Miller DA (1996) Allelopathy in forage crop systems. *Agronomy Journal* **88**, 854-859.

Miller DA, Shrivastava JP, Jackobs JA (1969) Alfalfa yield components in solid seedings. *Crop Science* **9**, 440-443.

Moate PJ, Clarke T, Davis LH, Laby RH (1997) Rumen gases and bloat in grazing dairy cows. *Journal of Agricultural Science* **129**, 459-469.

Monroy AF, Castonguay Y, Laberge S, Sarhan F, Vezina LP, Dhindsa RS (1993) A new cold-induced alfalfa gene is associated with enhanced hardening at subzero temperature. *Plant Physiology* **102**, 873-879.

Moot DJ, Brown HE, Teixeira EI, Pollock KM (2003) Crop growth and development affect seasonal priorities for lucerne management. *Legumes for dryland pastures. Proceedings of a New Zealand Grassland Association (Inc.) Symposium held at Lincoln University, 18-19 November, 2003*, 201-208.

Mugwira LM, Haque I (1993) Screening forage and browse legumes germplasm to nutrient stress I: tolerance of *Medicago sativa* L. to aluminum and low phosphorus in soils and nutrient solutions. *Journal of Plant Nutrition* **16**, 17-35.

Munns DN (1965) Soil acidity and growth of a legume. III. Interaction of lime and phosphate on growth of *Medicgo sativa* L. in relation to aluminium toxicity and phosphate fixation. *Australian Journal of Agricultural Research* **16**, 757-766.

Musgrave DJ, Langer RHM (1977) Crown development of two diverse genotypes of lucerne. *New Zealand Journal of Agricultural Research* **20**, 453-458.

Nelson CJ, Smith D (1968a) Growth of birdsfoot treefoil and alfalfa: III. Changes in carbohydrate reserves and growth analysis under field conditions. *Crop Science* **8**, 25 - 28.

Nelson CJ, Smith D (1968b) Growth of birdsfoot trefoil and alfalfa. II. Morphological development and dry matter distribution. *Crop Science* **8**, 21 - 25.

Nicolodi C, Massacci A, Marco GD (1988) Water status effects on net photosynthesis in field-grown alfalfa. *Crop Science* **28**, 944-948.

Noquet C, Avice JC, Ourry A, Volenec JJ, Cunningham SM, Boucaud J (2001) Effects of environmental factors and endogenous signals on N uptake, N

partitioning and taproot vegetative storage protein accumulation in *Medicago sativa*. *Australian Journal of Plant Physiology* **28**, 279-287.

Norton MR, Lelievre F, Volaire F (2006) Summer dormancy in *Dactylis glomerata* L.: the influence of season of sowing and a simulated mid-summer storm on two contrasting cultivars. *Australian Journal of Agricultural Research* **57**, 565-575.

Nuttall WF, Cooke DA, Waddington J, Robertson JA (1980) Effect of nitrogen and phosphorus fertilizers on a bromegrass and alfalfa mixture grown under two systems of pasture management. I. Yield, percentage legume in sward, and soil tests. *Agronomy Journal* **72**, 289-294.

Nylander M, Svensson J, Palva ET, Welin BV (2001) Stress-induced accumulation and tissue-specific localization of dehydrins in *Arabidopsis thaliana*. *Plant Molecular Biology* **45**, 263-279.

Orloff S, Hanson B (2008) Conserving water through deficit irrigation of alfalfa in the intermountain area of California. *Forage and Grazinglands*, FG-2008-0421-2001-RS.

Ottman MJ (1999) Water stress and alfalfa production. In 'Handbook of Plant and Crop Stress 2nd Edition'. (Ed. M Pessarakli) pp. 879 - 889. (Marcel Dekker: New York).

Palmer TP (1967) Lucerne breeding in New Zealand. In 'The Lucerne Crop'. (Ed. RHM Langer). (Reed Publishing: Wellington).

Paquin R, Pelletier G (1981) Natural cold acclimatization in lucerne (*Medicago media* Pers.). 1. Variations in the free proline content of leaves and crowns. *Physiologie Vegetale* **19**, 103-117.

Paquin R, Pelletier H (1980) Influence of the environment on acclimatization of lucerne (*Medicago media* Pers.) to cold and its resistance to frost. *Canadian Journal of Plant Science* **60**, 1351-1366.

Patterson DT (1993) Effects of day and night temperature on goatsrue (*Galega officinalis*) and alfalfa (*Medicago sativa*) growth. *Weed Science* **41**, 38-45.

Pearce RB, Lee DR (1969) Photosynthetic and morphological adaptation of alfalfa leaves to light intensity at different stages of maturity. *Crop Science* **9**, 791-794.

Pearson CJ, Hunt LA (1972a) Effects of pretreatment temperature on carbon-dioxide exchange in alfalfa. *Canadian Journal of Botany* **50**, 1925-1930.

Pearson CJ, Hunt LA (1972b) Effects of temperature on primary growth of alfalfa. *Canadian Journal of Plant Science* **52**, 1007-1015.

Pecetti L, Romani M, Rosa Ld, Piano E (2008) Selection of grazing-tolerant lucerne cultivars. *Grass and Forage Science* **63**, 360-368.

Pelton RE, Marble VL, Wildman WE, Peterson G (1988) Fall grazing by sheep on alfalfa. *California Agriculture* **42**, 4-5.

Pennycooke JC, Cheng H, Stockinger EJ (2008) Comparative genomic sequence and expression analyses of *Medicago truncatula* and alfalfa subspecies *falcata* COLD-ACCLIMATION-SPECIFIC genes. *Plant Physiology* **146**, 1242-1254.

Peoples MB, Baldock JA (2001) Nitrogen dynamics of pastures: nitrogen fixation inputs, the impact of legumes on soil nitrogen fertility, and the contributions of fixed nitrogen to Australian farming systems. *Australian Journal of Experimental Agriculture* **41**, 327-346.

Peoples MB, Bowman AM, Gault RR, Herridge DF, McCallum MH, McCormick KM, Norton RM, Rochester IJ, Scammell GJ, Schwenke GD (2001) Factors regulating the contributions of fixed nitrogen by pasture and crop legumes to different farming systems of eastern Australia. *Plant and Soil* **228**, 29-41.

Peoples MB, Ladha JK, Herridge DF (1995) Enhancing legume N_2 fixation through plant and soil management. *Plant and Soil* **174**, 83-101.

Perry LJ, Jr., Larson KL (1974) Influence of drought on tillering and internode number and length in alfalfa. *Crop Science* **14**, 693-696.

Pradhan SK, Varade SB, Kar S (1973) Influence of soil water conditions on growth and root porosity of rice. *Plant and Soil* **38**, 501-507.

Pratley JE, Corbin EJ (1993) Cultural Practices. In 'Principles of Field Crop Production'. (Ed. JE Pratley) pp. 302-348. (Oxford University Press: Melbourne).

Pritchard K, Havilah E, McRae C, Thompson P (1991) Fodder Crops. In 'Feedbase 2000: a workshop to determine the priorities for research into soils, pastures and fodder crops in Australian dairy production systems'. (Eds. B Bartsch, W Mason) pp. 47-58. (Dairy Research and Development Corporation: East Melbourne, Victoria).

Pumphrey FV, Wilkins DE, Hane DC, Smiley RW (1987) Influence of tillage and nitrogen fertilizer on Rhizoctonia root rot (bare patch) of winter wheat. *Plant Disease* **71**, 125-127.

Qamar SFA, Cunningham SM, Volenec JJ (2006) Phosphate nutrition and defoliation effects on growth and root physiology of alfalfa. *Journal of Plant Nutrition* **29**, 1387-1403.

Quiros CF, Bauchan GR (1988) The Genus *Medicago* and the Origin of the *Medicago sativa* Complex. In 'Alfalfa and Alfalfa Improvement'. (Eds. AA Hanson, DK Barnes, RR Hill) pp. 93-124. (American Society of Agronomy: Madison, WI, USA).

Rawnsley RP, Donaghy DJ, Stevens DR (2007) What is limiting production and consumption of perennial ryegrass in temperate dairy regions of Australia and New Zealand? In 'Dairy Science 2007, Meeting the Challenges for Pasture-Based Dairying, Proceedings of the 3rd Dairy Science Symposium'. (Eds. DF Chapman, DA Clark, KL Macmillan, DP Nation) pp. 256-276. (The University of Melbourne: Melbourne, Vicrtoria).

Read J, McGowan T, Reed K, Ward G, Lowe K, Kaehne I (1991) Perennial Pastures. In 'Feedbase 2000: a workshop to determine the priorities for research into soils, pastures and fodder crops in Australian dairy production systems'. (Eds. B Bartsch, W Mason) pp. 35-46. (Dairy Research and Development Corporation: East Melbourne, Victoria).

Remus-Borel W, Castonguay Y, Cloutier J, Michaud R, Bertrand A, Desgagnes R, Laberge S (2010) Dehydrin variants associated with superior freezing tolerance in alfalfa (*Medicago sativa* L.). *TAG Theoretical and Applied Genetics* **120**, 1163-1174.

Retta A, Hanks RJ (1980) Corn and alfalfa production as influenced by limited irrigation. *Irrigation Science* **1**, 135-147.

Reynolds SG, Smith D (1962) Trend of carbohydrate reserves in alfalfa, smooth bromegrass, and timothy grown under various cutting schedules. *Crop Science* **2**, 333-336.

Richard C, Martin JG (1993) The influence of *Fusarium oxysporum* f.sp. *medicaginis* on total soluble-sugar concentration of infected alfalfa roots. *Canadian Journal of Plant Science* **73**, 647-649.

Ridley AM, Haines PJ, Wilson KF, Ellington A (1998) Phase farming using lucerne in crop rotations. In 'Proceedings of the 9th Australian Agronomy Conference'. Wagga Wagga, New South Wales. (Eds. D Michalk, J Pratley).

Robertson M (2006) 'Lucerne prospects: Drivers for widespread adoption of lucerne for profit and salinity management.' (Cooperative Research Centre for Plant-based Management of Dryland Salinity: Perth, Western Austrlaia).

Robison GD, Massengale MA (1968) Effect of harvest management and temperature of forage yield, root carbohydrates, plant density and leaf area relationships in alfalfa. *Crop Science* **8**, 147-151.

Rogers VE (1961) Lucerne variety trials at Deniliquin, N.S.W. *Australian Journal of Experimental Agriculture and Animal Husbandry* **1**, 60-66.

Rogers VE (1981) Comparison of lucernes when grown on a heavy clay soil infected by Phytophthora, and their response to attack by the spotted alfalfa aphid (*Therioaphis trifolii* f. *maculata*). *Australian Journal of Experimental Agriculture and Animal Husbandry* **21**, 63-67.

Rosen H (1957) A modified ninhydrin colorimetric analysis for amino acids. *Archives of biochemistry and biophysics* **67**, 10-15.

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In 'Methods in Molecular Biology: Bioinformatics Methods and Protocols'. (Eds. S Misener, SA Krawetz). (Humana Press: Totowa, NJ, USA).

Russelle MP (2001) Alfalfa. *American Scientist* **89**, 252-261.

Ryley MJ (2002) Diseases of lucerne: problems and identification. In 'The lucerne management handbook'. (Ed. K Bullen) pp. 16-28. (Queensland Department of Primary Industries: Brisbane, Queensland).

Salter R, Melton B, Wilson M, Currier C (1984) Selection in alfalfa for forage yield with three moisture levels in drought boxes. *Crop Science* **24**, 345-349.

Sammis TW (1981) Yield of alfalfa and cotton as influenced by irrigation. *Agronomy Journal* **73**, 323-329.

Sanderson MA, Jones RM (1993) Stand dynamics and yield components of alfalfa as affected by phosphorus fertility. *Agronomy Journal* **85**, 241-246.

Scott BJ (2000) Resurrection plants and the secrets of eternal leaf. *Annals of Botany* **85**, 159-166.

Sheaffer CC, Barnes DK, Warnes DD, Lueschen WE, Ford HJ, Swanson DR (1992) Seeding-year cutting affects winter survival and its association with fall growth score in alfalfa. *Crop Science* **32**, 225-231.

Sheaffer CC, Lacefield GD, Marble VL (1988) Cutting Schedules and Stands. In 'Alfalfa and Alfalfa Improvement'. (Eds. AA Hanson, DK Barnes, RRJ Hill) pp. 411-437. (American Society of Agronomy: Madison, WI, USA).

Sheehy JE, Popple SC (1981) Photosynthesis, water relations, temperature and canopy structure as factors influencing the growth of sainfoin (*Onobrychis viciifolia* Scop.) and lucerne (*Medicago sativa* L.). *Annals of Botany* **48**, 113-128.

Sheehy JE, Woodward FI, Jones MB, Windram A (1979) Microclimate, photosynthesis and growth of lucerne (*Medicago sativa* L.). I. Microclimate and photosynthesis. *Annals of Botany* **44**, 693-707.

Shih SC, Jung GA, Shelton DC (1967) Effects of temperature and photoperiod on metabolic changes in alfalfa in relation to cold hardiness. *Crop Science* **7**, 385-389.

Simon JC, Decau ML, Avice JC, Jacquet A, Meuriot F, Allirand JM (2004) Effects of initial N reserve status and residual leaf area after cutting on leaf area and organ establishment during regrowth of alfalfa. *Canadian Journal of Plant Science* **84**, 1059-1066.

Singh Y, Winch JE (1974) Morphological development of two alfalfa cultivars under various harvesting schedules. *Canadian Journal of Plant Science* **54**, 79-87.

Smethurst CF, Garnett T, Shabala S (2005) Nutritional and chlorophyll fluorescence responses of lucerne (*Medicago sativa*) to waterlogging and subsequent recovery. *Plant and Soil* **270**, 31-45.

Smethurst CF, Shabala S (2003) Screening methods for waterlogging tolerance in lucerne: comparative analysis of waterlogging effects on chlorophyll fluorescence, photosynthesis, biomass and chlorophyll content. *Functional Plant Biology* **30**, 335-343.

Smith SR, Jr., Bouton JH, Hoveland CS (1989) Alfalfa persistence and regrowth potential under continuous grazing. *Agronomy Journal* **81**, 960-965.

Smith SRJ, Bouton JH (1993) Selection within alfalfa cultivars for persistence under continuous stocking. *Crop Science* **33**, 1321-1328.

Soil Survey Staff (1990) 'Keys to Soil Taxonomy 4th edition. Soil Management Support Service Technical Monograph No. 19 ' (Soil Survey Staff: Blacksburg, VA, USA).

Staswick PE (1994) Storage proteins of vegetative plant tissues. *Annual Review of Plant Physiology and Plant Molecular Biology* **45**, 303-322.

Stout DG, Hall JW (1989) Fall growth and winter survival of alfalfa in interior British Columbia. *Canadian Journal of Plant Science* **69**, 491-499.

Summers CG, Gilchrist DG (1991) Temporal changes in forage alfalfa associated with insect and disease stress. *Journal of Economic Entomology* **84**, 1353-1363.

Suzuki M (1991) Effects of stand age on agronomic, morphological and chemical characteristics of alfalfa. *Canadian Journal of Plant Science* **71**, 445-452.

Ta TC, Macdowall FDH, Faris MA (1990) Utilization of carbon and nitrogen reserves of alfalfa in supporting N_2-fixation and shoot regrowth. *Plant and Soil* **127**, 231-236.

Tan HY, Ng TW, Liew OW (2007) Effects of light spectrum in flatbed scanner densitometry of stained polyacrylamide gels. *Biotechniques* **42**, 474-478.

Teixeira EI, Moot DJ, Brown HE (2008) Defoliation frequency and season affected radiation use efficiency and dry matter partitioning to roots of lucerne (*Medicago sativa* L.) crops. *European Journal of Agronomy* **28**, 103-111.

Teixeira EI, Moot DJ, Brown HE (2009) Modelling seasonality of dry matter partitioning and root maintenance respiration in lucerne (*Medicago sativa* L.) crops. *Crop and Pasture Science* **60**, 778-784.

Teixeira EI, Moot DJ, Brown HE, Fletcher AL (2007a) The dynamics of lucerne (*Medicago sativa* L.) yield components in response to defoliation frequency. *European Journal of Agronomy* **26**, 394-400.

Teixeira EI, Moot DJ, Brown HE, Pollock KM (2007b) How does defoliation management impact on yield, canopy forming processes and light interception of lucerne (*Medicago sativa* L.) crops? *European Journal of Agronomy* **27**, 154-164.

Teixeira EI, Moot DJ, Mickelbart MV (2007c) Seasonal patterns of root C and N reserves of lucerne crops (*Medicago sativa* L.) grown in a temperate climate were affected by defoliation regime. *European Journal of Agronomy* **26**, 10-20.

Teuber LR, Brick MA (1988) Morphology and Anatomy. In 'Alfalfa and Alfalfa Improvement'. (Eds. AA Hanson, DK Barnes, RR Hill) pp. 125-162. (American Society of Agronomy: Madison, WI, USA).

Teuber LR, Taggard KL, Gibbs LK, McCaslin MH, Peterson MA, Barnes DK (1998) Fall dormancy. In 'Standard test to characterize alfalfa cultivars'. (Ed. CC Fox) pp. A-1-2. (North American Alfalfa Improvement Conference: Bozeman, MT, USA).

Tindall TA, Hurst CJ (1988) Selected alfalfa cultivar response to potassium and phosphorus grown on calcareous soils. *Journal of Fertilizer Issues* **5**, 148-154.

Tomes GJ, King GW, McFarlane JD (1972) The influence of grazing management on regrowth of lucerne in late autumn and winter. *Australian Journal of Experimental Agriculture and Animal Husbandry* **12**, 485-487.

Tomkins JP, Hall MH (1991) Stimulation of alfalfa bud and shoot development with cytokinins. *Agronomy Journal* **83**, 577-581.

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets - procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350-4354.

Triplett EW, Albrecht KA, Oplinger ES (1993) Crop rotation effects on populations of *Bradyrhizobium japonicum* and *Rhizobium meliloti*. *Soil Biology & Biochemistry* **25**, 781-784.

Turan M, Kiziloglu FM, Ketterings QM (2009) Phosphorus management of lucerne grown on calcareous soil in Turkey. *Journal of Plant Nutrition* **32**, 516-535.

Ueno M, Smith D (1970) Influence of temperature on seedling growth and carbohydrate composition of three alfalfa cultivars. *Agronomy Journal* **62**, 764-767.

Undersander DJ (1987) Alfalfa (*Medicago sativa* L.) growth response to water and temperature. *Irrigation Science* **8**, 23-33.

Undersander DJ, Grau C, Cosgrove D, Doll J, Martin N (1998) 'Alfalfa stand assessment: Is the stand good enough to keep?, Extension Bulletin A3620.' (University of Wisconsin: Madison, WI, USA).

Van Handel E (1968) Direct microdetermination of sucrose. *Analytical Biochemistry* **22**, 280-283.

Vance CP, Heichel GH (1981) Nitrate assimilation during vegetative regrowth of alfalfa. *Plant Physiology* **68**, 1052-1056.

Vance CP, Heichel GH, Barnes DK, Bryan JW, Johnson LE (1979) Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. *Plant Physiology* **64**, 1-8.

Vance CP, Heichel GH, Phillips DA (1988) Nodulation and Symbiotic Dinitrogen Fixation. In 'Alfalfa and Alfalfa Improvement'. (Eds. AA Hanson, DK Barnes, RRJ Hill) pp. 229-257. (American Society of Agronomy: Madison, WI, USA).

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**, RESEARCH0034.

Vincent SG, Cunningham PR, Stephens NL, Halayko AJ, Fisher JT (1997) Quantitative densitometry of proteins stained with Coomassie Blue using a Hewlett Packard scanjet scanner and Scanplot software. *Electrophoresis* **18**, 67-71.

Volaire F (2002) Drought survival, summer dormancy and dehydrin accumulation in contrasting cultivars of *Dactylis glomerata*. *Physiologia Plantarum* **116**, 42-51.

Volenec JJ (1985) Leaf area expansion and shoot elongation of diverse alfalfa germplasms. *Crop Science* **25**, 822-827.

Volenec JJ (1988) Herbage growth and carbohydrate metabolism of diploid and tetraploid alfalfa. *Crop Science* **28**, 128 - 132.

Volenec JJ (1999) Physiological Control of Alfalfa Growth and Yield. In 'Crop Yield: Physiology and Processes'. (Ed. DL Smith) pp. 422-442. (Springer: New York, NY, USA).

Volenec JJ, Boyce PJ, Hendershot KL (1991) Carbohydrate metabolism in taproots of *Medicago sativa* L. during winter adaptation and spring regrowth. *Plant Physiology* **96**, 786-793.

Volenec JJ, Cherney JH, Johnson KD (1987) Yield components, plant morphology, and forage quality of alfalfa as influenced by plant population. *Crop Science* **27**, 321-326.

Volenec JJ, Cunningham SM, Haagenson DM, Berg WK, Joern BC, Wiersma DW (2002) Physiological genetics of alfalfa improvement: past failures, future prospects. *Field Crops Research* **75**, 97-110.

Volenec JJ, Ourry A, Joern BC (1996) A role for nitrogen reserves in forage regrowth and stress tolerance. *Physiologia Plantarum* **97**, 185-193.

Vough LE, Marten GC (1971) Influence of soil moisture and ambient temperature on yield and quality of alfalfa forage. *Agronomy Journal* **63**, 40-42.

Wade LJ, McLaren CG, Samson BK, Regmi KR, Sarkarung S (1996) The importance of environmental characterization for understanding genotype by environment interactions. In 'Plant adaptation and crop improvement.'. (Eds M Cooper, GL Hammer) pp. 549-562. (CAB International: Wallingford).

Wang E, Cresswell H, Yu Q, Verburg K (2008) Summer forage cropping as an effective way to control deep drainage in south-eastern Australia - a simulation study. *Agriculture, Ecosystems & Environment* **125**, 127-136.

Ward PR, Micin SF (2006) The capacity of dryland lucerne for groundwater uptake. *Australian Journal of Agricultural Research* **57**, 483-487.

Wiersma DW, Grau CR, Undersander DJ (1995) Alfalfa cultivar performance with differing levels of resistance to phytophthora and aphanomyces root rots. *Journal of Production Agriculture* **8**, 259-264.

Wiersma DW, Hartman WG (2000) Alfalfa Variety Performance Database. (College of Agricultural and Life Sciences, University of Wisconsin: Madison, WI, USA).

Wilding MD, Stahmann MA, Smith D (1960) Free amino acids in alfalfa as related to cold hardiness. *Plant Physiology* **35**, 726-732.

Wolf DD, Blaser RE (1972) Photosynthesis of plant parts of alfalfa as influenced by canopy and light. *Crop Science* **12**, 23-26.

Wolfraim LA, Dhindsa RS (1993) Cloning and sequencing of the cDNA for *cas17*, a cold acclimation-specific gene of alfalfa. *Plant Physiology* **103**, 667-668.

Wolfraim LA, Langis R, Tyson H, Dhindsa RS (1993) cDNA sequence, expression, and transcript stability of a cold acclimation-specific gene, *cas18*, of alfalfa (*Medicago falcata*) cells. *Plant Physiology* **101**, 1275-1282.

Wright GC, Rao RCN, Basu MS (1996) A physiological approach to the understanding of genotype by environment interactions - a case study on improvement of drought adaptation in groundnut. In 'Plant adaptation and crop improvement.'. (Eds M Cooper, GL Hammer) pp. 365-381. (CAB International: Wallingford).

Wynn-Williams RB, Rea MB, Purves RG, Hawthorne BT (1991) Influence of winter treading on lucerne growth and survival. *New Zealand Journal of Agricultural Research* **34**, 271-275.

Yamamoto S, Kaneko T, Mino Y (1996) Starch metabolism in the tap root of alfalfa after defoliation. *Grassland Science* **41**, 302 - 306

Zadoks JC, Chang TT, Konzak CF (1974) A decimal code for the growth stages of cereals. *Weed Research* **14**, 415-421.

Zeid IM, Shedeed ZA (2006) Response of alfalfa to putrescine treatment under drought stress. *Biologia Plantarum* **50**, 635-640.

Zook DM, Erwin DC, Stolzy LH (1986) Anatomical, morphological, and physiological responses of alfalfa to flooding. *Plant and Soil* **96**, 293-296.

APPENDIX

Published peer-reviewed science publications from this research